INVESTIGATING THE EFFECTS OF *IN UTERO* ARSENIC EXPOSURE ON BIRTH OUTCOMES AND INFLUENZA A VIRUS VACCINE IMMUNOGENICITY IN THE OFFSPRING

by

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Abstract

Background: Arsenic is a common groundwater contaminant that has a variety of health impacts. Currently, the drinking water standard established by the World Health Organization is 10 μg/L or ppb. However, over 140 million people consume drinking water with arsenic levels that exceed the standard. Arsenic is a known immunotoxin, and has been linked to increased morbidity and mortality from respiratory infections like the influenza virus.

Objective: The goal of this study was to investigate the effects of *in utero* arsenic exposure on birth outcomes and influenza A virus (IAV) vaccine immunogenicity in the offspring.

Methods: Female C57BL/6J mice were treated with either 0 or 100 ppb of arsenic through their drinking water. After 1 to 2 weeks of exposure, females were mated with males. Throughout pregnancy, females were continuously given treated water to model *in utero* exposure. At 2 and 5 weeks of age, pups were immunized by intraperitoneal injection with an inactivated mouse-adapted (ma) 2009 H1N1 IAV. Vaccine immunogenicity was evaluated by enzyme-linked immunosorbent assays (ELISAs) and neutralizing antibody assays. At 8 weeks of age, the pups were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009

H1N1 IAV. Body weight and temperature of infected offspring were recorded daily, and tissue samples were collected throughout the IAV disease course.

Results: *In utero* exposure caused a significant decrease in fetal weight at gestation day 17, as well as pup weight at 1 week of age. Although pre-challenge immunogenicity measurements including anti-ma2009 H1N1 IAV IgG titers and neutralizing antibody titers showed that *in utero* arsenic exposure does not adversely alter the antibody-mediated response initiated after vaccination, *in utero* exposure to arsenic did inhibit weight gain following challenge with the drift variant IAV.

Conclusion: The results from this study confirm that arsenic is a developmental toxin. Future research will be directed towards understanding how arsenic differentially targets the cellular and humoral arms of the immune system.

Acknowledgements

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PART 1: INTRODUCTION AND BACKGROUND

1.1: Introduction

Arsenic is a naturally occurring element found in the earth's crust, and therefore is a common contaminant of drinking water sources in many countries including Bangladesh, Chile, India, and the United States (World Health Organization 2018). Individuals are most commonly exposed to arsenic through their drinking water; however, they can also be exposed to the metalloid by the ingestion of foods irrigated by contaminated water or through industrial processes such as mining (as reviewed by Ratnaike 2003).

The current standard for arsenic in drinking water established by the World Health Organization and the U.S. Environmental Protection Agency is 10 μg/L (ppb). However, more than 140 million individuals living in over 50 different countries consume drinking water with arsenic levels that far exceed the standard (Figure 1; World Health Organization 2018; U.S. Environmental Protection Agency 2018). For example, from 1958 to 1971, parts of Northern Chile experienced a period of prolonged exposure, with

arsenic levels 80 times greater than the drinking water standard (Steinmaus et al. 2013).

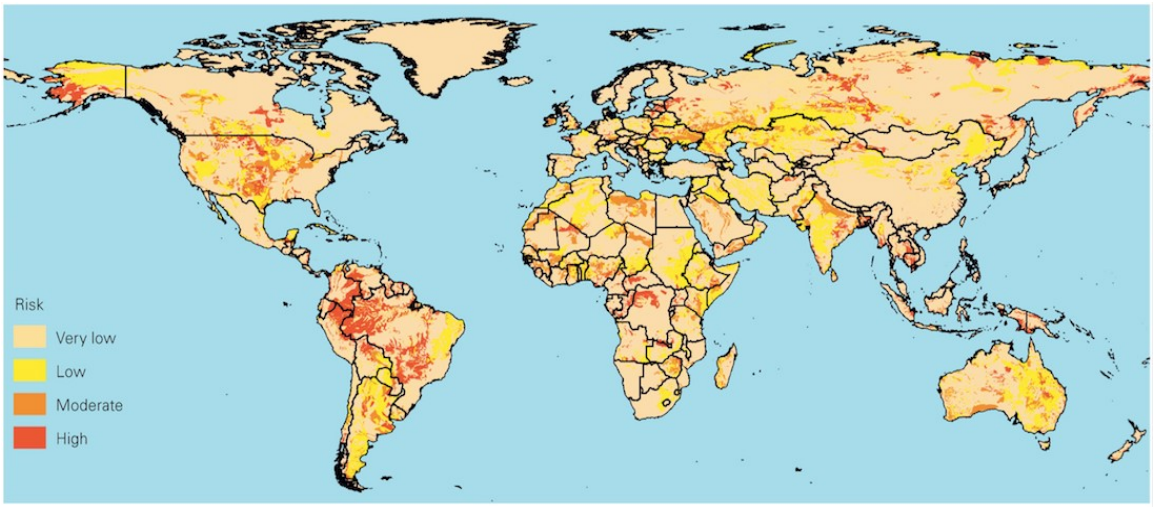

Figure 1: Estimated risk of arsenic contamination in drinking water above the World Health Organization standard of 10 μg/L (Schwarzenbach et al. 2010).

Having a better understanding of the health effects associated with arsenic is critical for protecting public health since millions of people are exposed to high levels of the metalloid in their drinking water. Specifically, a greater emphasis needs to be placed on investigating how *in utero* exposure to arsenic can alter pregnancy outcomes, as well as immune system function in the offspring.

1.2: Background

1.2.1: Properties of Arsenic

Arsenic can be found in several forms. When arsenic combines with carbon or hydrogen, it forms an organic arsenic compound. When arsenic is bound to oxygen, chlorine, or sulfur, it forms an inorganic arsenic compound (Agency of Toxic Substances and Disease Registry 2011a). Organic arsenic compounds are commonly found in seafood, while inorganic arsenic compounds are responsible for the contamination of groundwater sources (Schoof et al. 1999). Previously, exposure to arsenic was linked to the use of arsenic-containing wood preservatives, as well as pesticides and paints. Since its elimination in these products, exposure to inorganic arsenic occurs primarily through the consumption of contaminated drinking water (Centers for Disease Control and Prevention 2009). Individuals can also be exposed to inorganic arsenic through the inhalation of arsenic containing coal fumes and through the ingestion of poultry treated with arsenical growth promoters such as roxarsone and nitrasone (Agency for Toxic Substances and Disease Registry 2011a; U.S. Food and Drug Administration 2017).

In the environment, inorganic arsenic exists as arsenite (As_{III}) or arsenate (As_{V}; Hughes 2002). In the human body, arsenate is metabolized to arsenite, which is subsequently methylated to form monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), two organic arsenic compounds that are more readily excreted in comparison to their inorganic counterparts

(Drobna et al. 2009; Vahter and Concha 2001). However, both MMA and DMA play important roles in arsenic toxicity because of their cytotoxic and genotoxic properties, as well as their action as a tumor promoter, enzyme inhibitor, and teratogen (as reviewed by Dangleben, Skibola, and Smith 2013; Drobna et al. 2009; Hegedus et al. 2008; Mass et al. 2001).

Acute exposure to arsenic can lead to health problems including nausea, abdominal pain, and diarrhea. Numbness, muscle cramping, and death can also occur upon exposure to high doses of arsenic (as reviewed by Ratnaike 2003; George et al. 2014). Chronic exposure is linked to cancer of the lungs, bladder, and liver, skin lesions, peripheral neuropathy, and anemia (as reviewed by Naujokas et al. 2013). In addition, chronic exposure to arsenic is associated with developmental disorders, immune dysfunction, and adverse pregnancy outcomes (as reviewed by Attreed, Navas-Aciens, and Heaney 2017; Concha et al. 1998; Biswas et al. 2008). Currently, arsenic is classified as a human carcinogen by the International Agency for Research on Cancer and the U.S. Environmental Protection Agency (Agency for Toxic Substances and Disease Registry 2011b; U.S. Environmental Protection Agency 2016).

1.2.2: Case Studies of Arsenic Exposure

Most countries have drinking water sources that are contaminated with low levels of arsenic; however, case studies like Bangladesh and Chile

represent extreme situations where individuals consumed drinking water with arsenic levels far above the established standard of 10 μg/L for a prolonged period of time.

1.2.2.1: Bangladesh

In the 1940s, tube wells were installed in Bangladesh, a highly populated and developing country, to provide inhabitants with a steady supply of drinking water free of microorganisms; in the past, consumption of contaminated surface water led to gastrointestinal problems, causing a significant amount of mortality for the inhabitants of Bangladesh. By the early 1990s, millions of tube wells were constructed, providing drinking water to 80 percent of the population (Smith, Lingas, and Rahman 2000).

However, in 1993, it was discovered that the drinking water from the tube wells was contaminated with high levels of arsenic. The concentration of arsenic in the drinking water ranged from 50 μg/L to 300 μg/L, 5 to 30 times the standard established by the World Health Organization, depending on the location of the well. Some of the health effects experienced by the inhabitants of Bangladesh included skin lesions, cancer of the bladder, kidney, and lungs, neurological problems, cardiovascular disease, and respiratory tract infections (Smith, Lingas, and Rahman 2000; Spivey 2011; Rahman et al. 2011).

Even though the contaminated drinking water was identified in 1993, it is still an issue for Bangladesh. Although monitoring programs have been set up, most communities are still drinking from the contaminated tube wells since it is their only source of clean water. It is estimated that over 75 million inhabitants of Bangladesh were or are presently exposed to high levels of arsenic in their drinking water (Smith, Lingas, and Rahman 2000).

1.2.2.2: Northern Chile

Before 1958, the drinking water for Northern Chile was supplied by springs located in the Andes Mountains, which contained moderately high levels of inorganic arsenic, with concentrations around 90 μg/L. In 1958, Region II of Chile switched its drinking water source to the Holajar and Toconce rivers (Marshall et al. 2007). This resulted in drinking water with arsenic levels around 860 μg/L, over 80 times the standard established by the World Health Organization. The drinking water remained contaminated with arsenic until the installation of a water treatment plant in 1971, which led to a significant decline in arsenic levels. It is estimated that over 250,000 inhabitants experienced the 13-year period of highly contaminated drinking water (Steinmaus et al. 2013; Marshall et al. 2007).

Northern Chile provides a great example to study how chronic exposure to arsenic results in adverse health outcomes since the arsenic levels were documented before, during, and after switching the drinking

water source. Compared to other regions of Chile that sustained significantly lower arsenic levels in their drinking water, Region II inhabitants had an increased risk of mortality from bladder and lung cancer. A study also suggested that mortality was greater for those inhabitants born during the 13-year period of highly contaminated drinking water since deaths due to bladder and lung cancer continued to be elevated until the late 1990s (Steinmaus et al. 2013; Marshall et al. 2007; Smith et al. 1998).

In addition to increased mortality from bladder and lung cancer, Region II of Chile had higher rates of deaths caused by acute myocardial infarction compared to other regions of Chile; when mortality from myocardial infarction was stratified by age, men who were born during the highest exposure period, experiencing arsenic-contaminated drinking water both *in utero* and during early childhood, had the highest rate ratio (Yuan et al. 2007). Studies looking at the effects of arsenic exposure *in utero* and during early life have also noted an increase in mortality from bronchiectasis and chronic obstructive pulmonary disease in children born in Region II during the 13-year period (Smith et al. 2005). Additional epidemiology studies conducted in Chile have indicated that high levels of arsenic resulted in increased mortality from infectious diseases including pulmonary tuberculosis (as reviewed by Dangleben, Skibola, and Smith 2013; Smith et al. 2011).

1.2.3: Arsenic Exposure and Birth Outcomes

Because of the prevalence of arsenic-contaminated drinking water, several epidemiology studies have been conducted to investigate the effects of the metalloid on pregnancy outcomes. A cohort study in Bangladesh looked at the relationship between arsenic levels in drinking water and birth outcomes. Results showed that arsenic concentrations greater than 50 μg/L significantly increased the risk for spontaneous abortion. The study also found a dose-dependent relationship between arsenic exposure and risk of infant death (Rahman et al. 2007). A cross-sectional study looking at the effects of contaminated tube wells in Bangladesh found similar results when examining the relationship between arsenic exposure during pregnancy and risk for adverse outcomes including spontaneous abortion and infant death (Milton et al. 2005). Another cross-sectional study compared pregnancy outcomes between women exposed and unexposed to arsenic. Women who were exposed to water with arsenic levels over 50 μg/L had significantly higher rates of adverse outcomes including preterm birth and stillbirth (Ahmad et al. 2001).

Additional studies have been conducted to assess the effects of *in utero* arsenic exposure on infantile infection rates. A prospective cohort study done in the United States looked at how *in utero* arsenic exposure influenced the number and severity of infections during the first 4 months of life. The study found a relationship between maternal urinary arsenic concentrations and

the number of respiratory tract infections in the infant including influenza and respiratory syncytial virus (relative risk=1.6, 95% confidence interval=1.0 to 2.5). This study also discovered that arsenic levels were highly correlated with respiratory symptoms such as wheezing, coughing, fever, and diarrhea (relative risk=4.0, 95% confidence interval=1.0 to 15.8; Farzan et al. 2013). A second prospective cohort study done in Bangladesh found similar results; *in utero* exposure to arsenic-contaminated drinking water increased the risk and severity of lower respiratory tract infections in infants during the first year of life (relative risk=1.69, 95% confidence interval=1.36 to 2.09; Rahman et al. 2011).

All of these studies determined that levels of arsenic above 50 μg/L have adverse consequences including an increased risk for spontaneous abortion, preterm birth, and stillbirth. These studies also indicated that *in utero* arsenic exposure could lead to greater infant morbidity and mortality caused by respiratory infections and symptoms like diarrhea. The increased risk for infantile infection and death could be related to the metalloid's immunosuppressive property (Soto-Pena et al. 2006; Milton et al. 2017). In addition, because arsenic easily crosses the placenta during early gestation to reach the fetus, neonates are especially vulnerable to exposure since they are undergoing rapid development and have only the initial components of an immune system (Vahter 2009; Hayward 1983).

1.2.4: Effects of Arsenic on the Immune System

In addition to being a known human carcinogen, arsenic also alters the function of the immune system (as reviewed by Attreed, Navas-Aciens, and Heaney 2017; as reviewed by Ratnaike 2003; Wu et al. 2003). The role of the immune system is to protect the body by the neutralization and elimination of antigens. The immune system is divided into innate and adaptive immunity. Innate immunity refers to non-specific defense mechanisms that immediately go into action upon the presence of antigens in the body. Adaptive immunity is a specialized immune response targeted for specific antigens that the body recognizes from a previous exposure. Both innate and adaptive immunity can be further divided into cellular and humoral immunity; cellular immunity involves different cells that can destroy antigens like T cells and lymphocytes, while humoral immunity refers to a response mediated by antibodies (as reviewed by Lee, Liao, and Yu 2011; Turvey and Broide 2010).

Studies have revealed that arsenic has numerous effects on the immune system. In animal and human studies, arsenic lowered T-cell proliferation and diminished the phagocytic activity of the macrophage (Burns and Munson 1993; Banerjee et al. 2009; Lemarie et al. 2006). In addition, nitric oxide release was lower and CD4+ cells from the spleen were higher for arsenic-exposed populations (Kumagai and Pi 2004; Gera et al. 2017). Furthermore, arsenic increased tumor burden in the lungs, liver, and

bladder in both animals and humans (Chen and Ahsan 2004; Wei et al. 2002; Cui et al. 2006).

Human population studies have identified notable effects of arsenic on the immune system. Individuals exposed to arsenic through their drinking water have differences in expression for various genes responsible for immune function compared to those unexposed to arsenic-contaminated drinking water. In a genome-wide expression study of peripheral blood mononuclear cells, arsenic exposure was linked to decreased expression of MHC class II molecules and inflammatory genes (Andrew et al. 2008). In addition, arsenic was associated with the down regulation of cytokines including interleukin 1 beta, which plays an important role in cell signaling, as well as interleukin 2, which is necessary for a cell-mediated immune response (as reviewed by Attreed, Navas-Aciens, and Heaney 2017; Argos et al. 2006; Ahmed et al. 2014).

Arsenic has also been shown to change immune cell populations in humans. Studies have revealed impaired lymphocyte function and proliferation, as well as decreased secretion of tumor necrosis factor alpha and various interleukins (Biswas et al. 2008). Other studies have indicated that exposure to arsenic increases the number of eosinophils and decreases the number of monocytes compared to unexposed individuals. Macrophage dysfunction, which involved reduced adhesion, lower nitric oxide production, and diminished phagocytic activity was also noted for individuals exposed to

arsenic (Banerjee et al. 2009). In addition, blood samples from children exposed to high levels of arsenic through their drinking water have shown an increase in the release of granulocyte-macrophage colony stimulating factor, resulting in chronic inflammation. This study in children has also identified lower CD4+ cell counts, resulting in a decreased CD4 to CD8 ratio, which may be an early indication of arsenic-mediated immunosuppression (Soto-Pena et al. 2006).

Arsenic has also proven to have effects on humoral immunity, which involves an immune response modulated by antibodies. Immunoglobulins or antibodies are proteins that recognize and bind to material that is foreign to the body (Solomon and Weiss 1995). A study conducted in Bangladesh revealed significantly higher global IgG, IgA, and IgE levels in the serum for individuals suffering from respiratory conditions like asthma and bronchitis who were chronically exposed to arsenic-contaminated drinking water. High levels of immunoglobulins could be an indication of a chronic infection, cancer, or an autoimmune disease (Islam et al. 2007). Another study found elevated global IgG levels in the serum of pregnant women, but not in the umbilical cord serum, suggesting that arsenic inhibits the transport of IgG to the fetus (as reviewed by Attreed, Navas-Aciens, and Heaney 2017; Ser et al. 2015).

1.2.5: Arsenic and Vaccine Immunogenicity

Vaccinations provide protection by priming the immune system to face future antigens. When a vaccine is administered, the weakened or inactivated antigen enters the body and initiates an immune response. The vaccine predominantly targets the humoral arm of the adaptive immune system to create antibodies against an antigen such as the influenza virus. After the vaccine is given, an individual is protected from a given antigen because their body has developed specific antibodies that will go into action upon the presence of the antigen (Clem 2011).

There are several types of vaccines in use. Live-attenuated vaccines contain a weakened antigen that does not cause disease in individuals with functioning immune systems; examples of live-attenuated vaccines include the rubella and mumps vaccines. Inactivated vaccines use dead viruses or bacteria to stimulate an immune response, consequently requiring multiple doses to build up immunity; an example of an inactivated vaccine is the polio vaccine. Toxoid vaccines protect against bacterial diseases that produce toxins in the body by using a weakened toxin or toxoid; examples of toxoid vaccines include the diphtheria and tetanus vaccines. Subunit vaccines contain only parts of a virus or bacteria, making side effects less common; the whooping cough vaccine is an example of a subunit vaccine. Conjugate vaccines protect against bacteria that have an outer coating of polysaccharides, which makes the antigen difficult to recognize by an

immature immune system; the *Haemophilus influenzae* type B vaccine given to children is an example of a conjugated vaccine (Clem 2011; Centers for Disease Control and Prevention 2013).

Studies have suggested that arsenic can affect vaccine immunogenicity. A study conducted in Bangladesh found that children exposed to arsenic-contaminated drinking water had increased plasma concentrations of global IgG and IgE compared to children unexposed to arsenic. This same study identified decreasing levels of mumps-specific immunoglobulins that corresponded to increasing levels of arsenic, implying that the metalloid impairs the vaccine-mediated antigen-specific antibody response (Raqib et al. 2017).

Another study looking at participants of the National Health and Nutrition Examination Survey (NHANES) discovered that increased arsenic exposure, measured through metabolites in the urine, was correlated with higher total anti-hepatitis A antibodies for individuals who previously received immunization for the virus. This study also noted greater seropositive status of hepatitis A with increasing levels of arsenic exposure, suggesting that the metalloid alters the immune response induced by the vaccine. However, this study was not able to determine whether arsenic increased the rate of seroconversion for individuals previously immunized with the hepatitis A vaccine (Cardenas et al. 2016).

A similar cross-sectional study using participants of NHANES examined the association between arsenic and varicella zoster virus IgG seroprevalence. Using metabolites in the urine to determine arsenic exposure, the study found an inverse relationship between total urinary arsenic and negative varicella zoster virus IgG for both vaccinated and non-vaccinated individuals. These results suggest that the immunosuppressive properties of arsenic can decrease both natural and vaccine-mediated immunity to the varicella zoster virus (Cardenas et al. 2015).

A different study conducted on Bangladeshi children looked at how the antibody response to vaccines was modified by arsenic exposure. The study discovered that arsenic in the drinking water did not negatively alter the antibody response to the cholera vaccine, as well as the diphtheria or tetanus vaccines. In addition, this study did not find any differences in the levels of measles-specific immunoglobulins between exposed and unexposed children (Saha et al. 2013).

1.2.6: Impact of Influenza

Influenza is a highly contagious respiratory infection caused by an influenza virus. Symptoms can vary widely but typically include fever, muscle aches, nasal congestion, and a persistent cough. Each year in the United States, it is estimated that the number of influenza cases ranges from 9.3 million to 49.0 million, and the number of deaths attributed to influenza

ranges from 12,000 to 79,000 (Centers for Disease Control and Prevention 2019a). Vulnerable populations including children under the age of 5, adults over the age of 65, and pregnant women are at the greatest risk for developing influenza-related complications such as pneumonia and multi-organ inflammation. To prevent the spread of influenza, as well as to decrease the severity of the illness, the Centers for Disease Control and Prevention recommends that every individual over the age of 6 months should receive the flu vaccine each year (Centers for Disease Control and Prevention 2018b). The seasonal influenza vaccine is available in many forms including an inactivated vaccine, a live-attenuated nasal spray, and an adjuvant vaccine; the type of influenza vaccine administered depends on a variety of factors including age, immune status, and medical history (Centers for Disease Control and Prevention 2018a; Chung et al. 2019). The influenza virus can be spread through a variety of different ways, but exposure to environmental contaminants such as arsenic could increase an individual's susceptibility to infection by altering the immune response (Liao et al. 2011).

The influenza virus is a RNA virus that encompasses three different subtypes: influenza A, influenza B, and influenza C. The three subtypes differ in host range and pathogenicity, with influenza A primarily infecting mammals including humans (Taubenberger and Morens 2008). Influenza A is further characterized by two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), that project from the virion. Although there are 16

HA and 9 NA glycoproteins, only 3 subtypes of HA and 2 subtypes of NA have been known to cause human influenza epidemics (Taubenberger and Morens 2008; Bouvier and Palese 2008).

There have been several influenza pandemics over the past century including the 1918 H1N1 Spanish Flu and the 1957 H2N2 Asian Flu (Potter 2001; Saunders-Hastings and Krewski 2016). The most recent pandemic was the 2009 Swine-Origin Pandemic. In April of 2009, a novel H1N1 influenza virus was isolated from humans in California and Mexico. The composition of the virus resulted from the exchange of gene segments between the North American H3N2 and H1N2 swine viruses, and the Eurasian avian-like swine viruses (Figure 2; Garten et al. 2009; Neumann, Noda, and Kawaoka 2009). Overall, 214 countries and territories had confirmed cases of the 2009 H1N1 influenza. In the United States alone, the number of cases ranged from 43 million to 89 million, and the number of deaths ranged from 8,870 to 18,300 (Centers for Disease Control and Prevention 2010). In addition, 80 percent of the deaths attributed to the 2009 H1N1 pandemic occurred in individuals under the age of 65; during a typical influenza epidemic, over 80 percent of deaths occur in individuals over the age of 65 (Dawood et al. 2012).

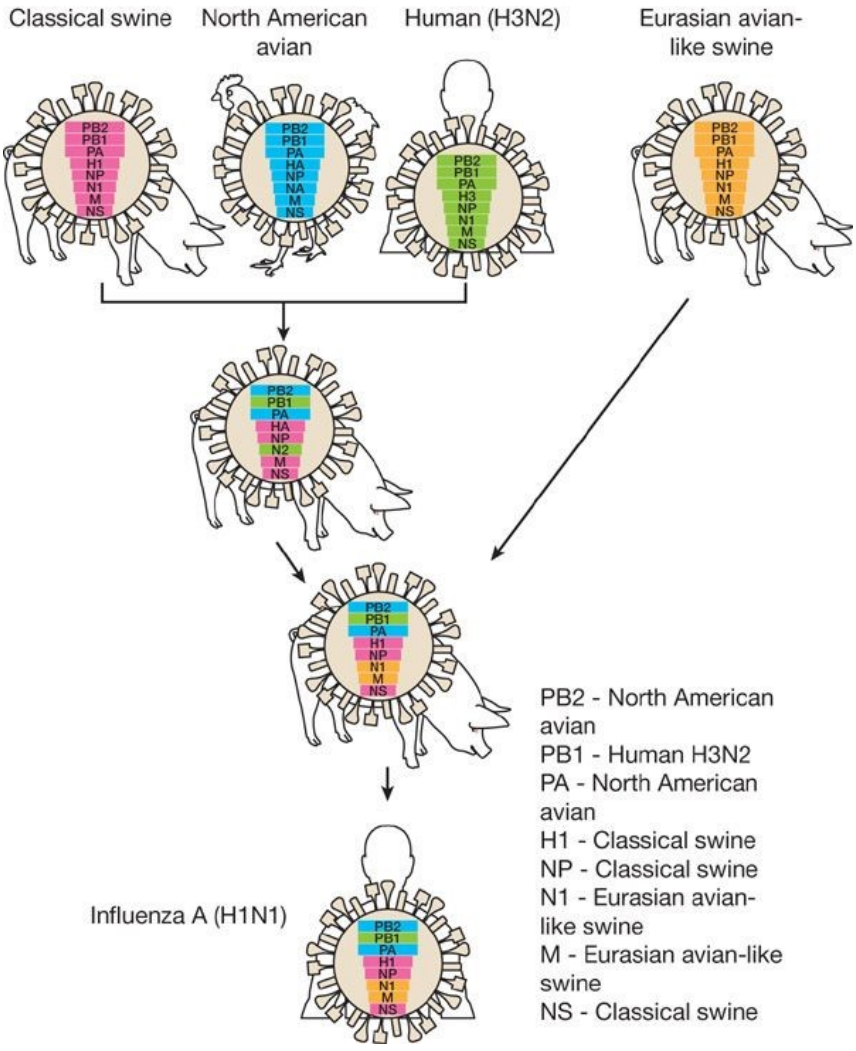

Figure 2: Genesis of swine-origin H1N1 influenza viruses (Neumann, Koda, and Kawaoka 2009).

1.2.7: Effects of Arsenic on Influenza Susceptibility

Some studies have linked arsenic exposure with an increased risk of developing respiratory infections such as influenza. One study exposed mice to arsenic both *in utero* and during postnatal life to determine if exposure to the metalloid alters the inflammatory response to H3N1 influenza A virus

infection. The results of the study indicated that *in utero* and early life arsenic exposure reduces the inflammatory response to influenza A virus by increasing the number of neutrophils, lymphocytes, and macrophages in bronchoalveolar lavage fluid. This study also discovered that arsenic exposure was associated with increased viral titers for influenza A virus. In addition, both male and female mice exposed to arsenic had notable adverse differences in lung structure and airway resistance, similar to those with bronchiectasis (Ramsey et al. 2013a). The results from this study demonstrated how arsenic exposure could potentially result in greater susceptibility to respiratory problems like influenza and bronchiectasis. In addition, the results suggest that exposure to arsenic *in utero* and during early life could effect the development of airway structure, resulting in an exacerbated inflammatory response to respiratory infections acquired in the future.

A study conducted in Taiwan, West Bengal, and the United States used a mathematical model to determine whether arsenic exposure decreases lung function and increases influenza susceptibility. It was discovered that exposure to arsenic decreases forced expiratory volume in one second, a respiratory metric that measures lung function and detects respiratory diseases like pulmonary fibrosis and emphysema. The model also revealed that chronic arsenic exposure and influenza A virus infection poses a significant risk to decreased lung function (Liao et al. 2011).

Another study used an adult mouse model to investigate the overall effects of arsenic on H1N1 influenza A virus infection. Results from the study performed in male mice showed that mice exposed to arsenic had increased morbidity, measured through body weight, after infection with the influenza A virus compared to the control group; the mice that were given arsenic lost a significant amount of weight while the unexposed mice experienced moderate weight loss, but recovered several days post infection. The exposed group also had a significant increase in viral titer, as well as histological changes including hemorrhage and edema compared to the unexposed group. Furthermore, the study discovered reduced cytokine production and decreased dendritic cells in lymph nodes of arsenic-exposed mice (Kozul et al. 2009).

1.2.8: Gaps in the Research

Although the effects of arsenic have been extensively explored, there are still knowledge gaps that should be addressed. First, a more concrete understanding about how arsenic exposure influences the immune system is needed. Several studies have suggested that arsenic alters the immune system, but the mechanisms driving these effects are still unknown (Banerjee et al. 2009; Gera et al. 2017). Additional information and studies are also needed to develop a more explicit dose-response relationship between arsenic

exposure and quantifiable changes in the immune system such as cytokine and antibody production.

Another significant knowledge gap is understanding how *in utero* exposure to arsenic influences the development of the immune system during both infancy and early childhood. Many studies have looked at the relationship between arsenic exposure and pregnancy outcomes, but few studies have investigated the effects of *in utero* exposure on infectious disease susceptibility in the offspring (Ahmad et al. 2001; Rahman et al. 2007). Addressing this gap in knowledge is critical because children are particularly vulnerable since they are still undergoing development and have an immature immune system (as reviewed by Ygberg and Nilsson 2011; as reviewed by Simon, Hollander, and McMichael 2015). In addition, arsenic-contaminated drinking water is a prevalent issue, especially in developing countries where infectious diseases are the primary cause of death (Jones et al. 2008).

A third knowledge gap involves understanding how *in utero* exposure to arsenic alters vaccine efficacy. Studies have looked at how chronic arsenic exposure influences vaccine immunogenicity; however, no studies have investigated how *in utero* arsenic exposure affects the developing immune system and immunological memory responses in infants (as reviewed by Attreed, Navas-Aciens, and Heaney 2017; Raqib et al. 2017). Information on *in utero* arsenic exposure during important developmental windows for the

immune system could provide valuable knowledge to the field of public health.

PART 2: RESEARCH QUESTION AND SPECIFIC AIMS

2.1: Research Question

As mentioned previously, there are several knowledge gaps in the field of arsenic exposure, especially when looking at the metalloid as an immunotoxin. For my project, I used a mouse model to investigate how *in utero* arsenic exposure influences birth outcomes and affects influenza A virus vaccine immunogenicity in the offspring.

2.2: Specific Aim 1

The goal for Specific Aim 1 was to determine how *in utero* arsenic exposure influences birth outcomes in the mice. Outcomes including reproductive success, fetal resorption, neonatal outcomes, and pup size were measured and compared.

2.3: Specific Aim 2

The goal for Specific Aim 2 was to determine the effects of *in utero* arsenic exposure on early life influenza A virus vaccine immunogenicity and subsequent infection susceptibility during adulthood. After *in utero* exposure to arsenic, the pups were vaccinated during the juvenile life stage, and then challenged with the drift variant of the influenza A virus (Figure 3). The pups were monitored to measure severity of the infection through changes in

body weight, temperature, and clinical scores. In addition, immunoassays were performed on serum samples to assess the abundance and functionality of influenza A virus-specific immunoglobulins (Yu et al. 2002; Mazanec, Coudret, and Fletcher 1995; Edenborough, Gilbertson, and Brown, 2012; Betts et al. 2012).

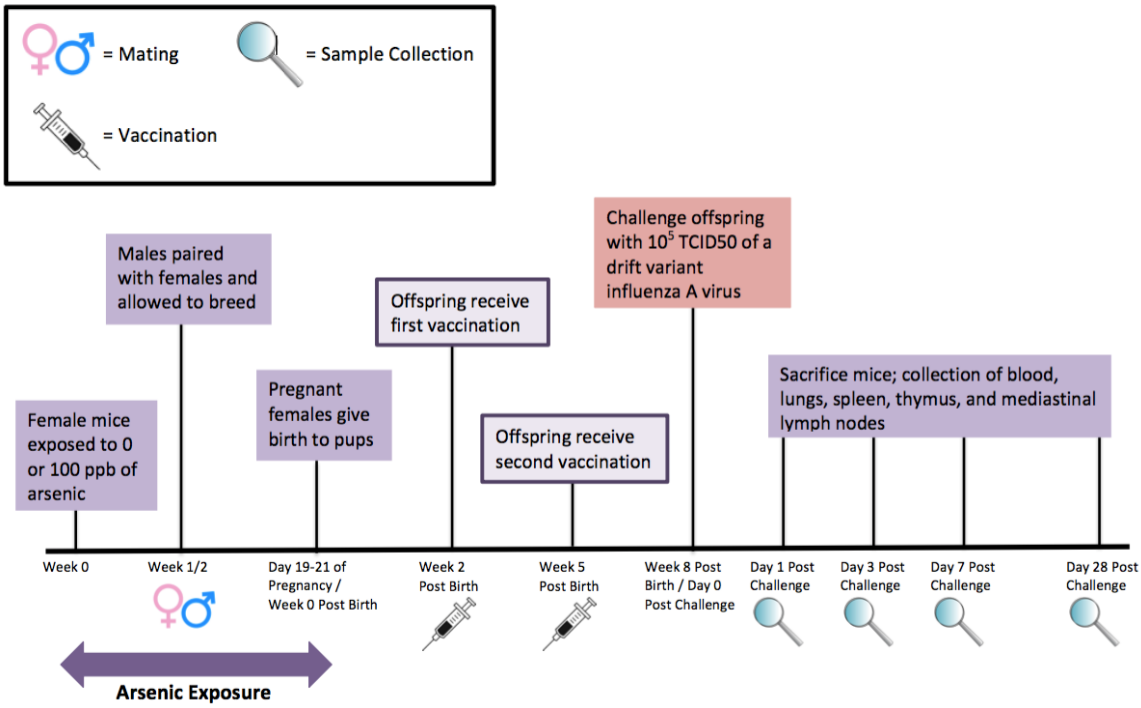

Figure 3: Experimental timeline for specific aim 2.

2.4: Public Health Significance

Arsenic-contaminated drinking water is a massive public health problem since millions of people are exposed to levels that far exceed the World Health Organization standard of 10 μg/L. Especially in countries such

as Chile and Bangladesh, extremely high arsenic levels are resulting in adverse health outcomes. In addition, arsenic is a harmful metalloid that is a known human carcinogen, as well as a developmental toxin and immunotoxin. Looking within the field of public health, arsenic-contaminated drinking water is also an environmental health problem. As the climate begins to change, drinking water resources will become limited and less accessible, particularly in developing countries. These countries will have to rely on alternative or unconventional resources such as tube wells, which may have high arsenic levels, depending on geological composition of the location, for a steady supply of drinking water. In addition, as the climate warms and precipitation patterns change, infectious diseases like influenza will become more prevalent, especially in developing countries where individuals with compromised immune systems will be challenged (Harper et al. 2011).

This innovative project was the first study to investigate how *in utero* arsenic exposure alters the offspring's humoral immune response to the influenza A virus vaccine. This project not only addressed knowledge gaps regarding arsenic-contaminated drinking water by providing more insight on how *in utero* exposure influences birth outcomes, but also uncovered how influenza A virus vaccine immunogenicity during early life changes based on *in utero* arsenic exposure.

The results from this study can be applied to better predict and prepare for the outcomes associated with *in utero* exposure to arsenic including an increased risk for influenza A virus infection. By having a greater understanding of how arsenic alters vaccine immunogenicity during early life, appropriate vaccination strategies can be applied to ensure protection from influenza-related morbidity and mortality, especially in young children exposed to arsenic *in utero*.

PART 3: MATERIALS AND METHODS

3.1: Animal Care

Adult (7 to 8 week old) male and female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). This specific strain of mouse was chosen because it is a commonly used model in influenza research (Bouvier and Lowen 2010; Thangavel and Bouvier 2014). For mating purposes, male mice were housed individually, while female mice were housed 2 per cage. All animals were housed in microisolator cages with corncob bedding, and kept in a facility with a 14-hour light/10-hour dark cycle. In addition, females were provided with extra cotton nestlets and plastic mouse houses for enrichment. Food and water were provided *ad libitum*. Johns Hopkins University Animal Care and Use Committee approved all animal procedures under protocol M017H323.

3.2: Arsenic Exposure

Arsenic stock solution was made by dissolving sodium meta-arsenite (Sigma-Aldrich) into arsenic-free drinking water (Crystal Geyser). The arsenic stock was diluted to create a solution with a final concentration of 100 μg/L or ppb.

Glass water bottles were filled with either arsenic treated water (100 ppb) or arsenic free water (0 ppb) and placed in the cages housing female

mice; females were exposed to treated water for 1 to 2 weeks prior to mating. Males were not provided with treated water, and instead were exposed to the facility drinking water. In addition, both males and females were given special animal feed containing low amounts of arsenic (Research Diets Inc.) to limit the intake of the metalloid from sources other than the drinking water. Twice a week, water bottles and food were replaced, and the amount of water and food consumed was recorded.

3.3: Timed Mating

Three days before timed mating, bedding was swapped between male and female cages to synchronize the estrous cycle to increase reproductive success (Byers et al. 2012). On the day of mating, 2 cohoused females were placed in the male cage, along with the treatment water bottle; the animals were allowed to mate overnight. The following morning, which marks gestation day 0, the females were taken out and placed back in their original cage with the treatment water bottle. The female mice were weighed on gestation day 10, and pregnancy was confirmed by a weight increase of ~2 grams from gestation day 0 (Heyne et al. 2015; Mader et al. 2009; Pauley and Washington 2017).

Mating occurred in 2 consecutive waves. After the 1st wave of mating, a 2nd wave of mating using non-pregnant females from the 1st wave of mating occurred during the following week. During both waves, the 2 cohoused

females were paired with the same male to increase successful mating and to decrease the chance of spontaneous abortion (Gangrade and Dominic 1984; Kumar and Dominic 1993; Eccard, Dammhahn, and Ylonen 2017). If it was discovered that 2 pregnant females were cohoused in 1 cage, 1 of the females was placed in her own cage between gestation day 15 and 16. Throughout pregnancy, the female dams were continuously exposed to either 0 ppb or 100 ppb arsenic water to model *in utero* exposure.

3.4: Gestation Day 17 Euthanasia

To further look at the effects of arsenic on birth outcomes, females were mated for a gestation day 17 euthanasia. Female mice (10 weeks old) were exposed to either arsenic treated water (100 ppb) or arsenic free water (0 ppb) for 2 weeks. The females were then mated with males using the same protocol employed previously. The females were weighed on gestation day 10, and pregnancy was confirmed by a weight increase of ~2 grams from gestation day 0 (Heyne et al. 2015; Mader et al. 2009; Pauley and Washington 2017). Throughout pregnancy, females were continuously given treated water to model *in utero* exposure.

On gestation day 17, 2 days prior to delivery of pups, the pregnant females were euthanized by the inhalation of isoflurane. Following blood collection from the dam, the entire uterine horn was removed from the abdomen and placed in ice-cold phosphate-buffered saline (PBS). The

number of offspring and fetal resorptions was noted, and a section of the right uterine horn was placed in zinc formalin for future histological analysis. Fetuses were then individually dissected out of the uterine horn, and the placenta and umbilical cord were removed from the fetus; the exterior membrane covering the placenta was clipped and the tissue was weighed (Klaunberg et al. 2004; Pritchett et al. 2005). Next, weight and crown-to-rump length of the fetus were measured and recorded. Lungs and heart of the fetus were isolated and weighed. All of the tissue was snap frozen in liquid nitrogen for future gene and protein expression work, as well as for arsenic speciation analysis.

3.5: Delivery and Weaning of Pups

Between gestation day 19 and 21, female dams delivered pups (Murray et al. 2010; McCarthy et al. 2018). On the day of delivery, exposure to arsenic stopped and treatment water bottles were all changed to 0 ppb water. One week post birth, the pups were counted and sexed, and any pups lost to cannibalism were noted. In addition, weight and crown-to-rump length were measured (Rhees and Atchley 2000).

At 4 weeks post birth, pups were weaned. The offspring were weighed and ear-punched. Males and females were housed up to 5 littermates per cage. The offspring were provided with facility drinking water and animal feed containing low amounts of arsenic *ad libitum*.

3.6: Synthesis of Influenza A Virus Vaccine

A mouse-adapted (ma) 2009 H1N1 strain of IAV synthesized by the lab of Dr. Andrew Pekosz (Johns Hopkins Bloomberg School of Public Health) was used for vaccination. Madin-Darby Canine Kidney (MDCK) cells were infected with viral media containing the ma2009 H1N1 IAV at a multiplicity of infection of 0.01. After a 4 day incubation at 32C, the infected cells were harvested and centrifuged to remove cell debris. Following centrifugation, beta-propiolactone was added to inactivate the virus. The infected cells were then purified by ultracentrifugation with a sucrose layer. The viral protein pellet was then resuspended in PBS containing calcium and magnesium to create the vaccine stock (Fink et al. 2018).

To determine the concentration of viral protein in the vaccine stock, a Pierce bicinchoninic acid (BCA) assay was performed (Thermo Scientific). After diluting by a factor of four and eight, the concentration of the viral protein was calculated by averaging the values and multiplying by the dilution factor.

3.7: Booster Strategy Vaccination

At 2 and 5 weeks post birth, the offspring were vaccinated with the inactivated ma2009 H1N1 IAV vaccine. On the day of vaccination, the vaccine stock was thawed and diluted with sterile PBS to a specific viral protein concentration. The offspring were then immunized with 100 μL/dose

of vaccine by intraperitoneal injection. Pups from the 1st wave of mating received 17.99 μg/dose for the first vaccination and 33.22 μg/dose for the second vaccination. Pups from the 2nd wave of mating received 32.16 μg/dose for the first vaccination and 36.19 μg/dose for the second vaccination (Table 1).

Table 1: Vaccine concentrations used to immunize offspring from the 1st and 2nd waves of mating.

Mating Wave	Vaccination 1	Vaccination 2
1	17.99 μg/dose	33.22 μg/dose
2	32.16 μg/dose	36.19 μg/dose

From the 2nd wave of mating, 2 male and 2 female offspring from each treatment group acted as unvaccinated controls. On the day of the first vaccination, the offspring were randomly designated as controls and tagged by ear-punch.

3.8: Pre-Challenge Blood Collection

At 21 days post vaccination 2 and before challenge with the drift variant IAV, blood was collected from the offspring. Samples were collected by facial bleeds through the submandibular vein (Golde, Gollobin, and Rodriguez 2005; Regan et al. 2016). These blood samples were spun down at 10,000 x gravity for 10 minutes to obtain serum.

3.9: Enzyme-Linked Immunosorbent Assay (ELISA)

To compare IgG, IgG1, and IgG2a/c titers against the ma2009 H1N1 IAV vaccine strain, ELISAs were performed on pre-challenge serum samples that were collected 21 days post vaccination 2 (Fink et al. 2018). Before running the assay, 96-well plates were coated with purified ma2009 H1N1 IAV protein diluted in ELISA coating buffer.

The following day, the plates were blocked with a mixture of milk powder and ELISA wash buffer, and incubated for 1 hour at 37C. During the incubation, the serum samples were serially diluted in a solution of wash buffer, milk power and bovine serum albumin (BSA). After removing the blocking buffer, the diluted samples were added to the plates in duplicate and incubated for 1 hour at 37C. Following the incubation, the plates were washed with wash buffer and the peroxidase-conjugated secondary antibody (Invitrogen), was added. After another 1 hour incubation at 37C, the plates were washed and 3, 3', 5, 5'-tetramethylbenzidine (Sigma) was added to each plate to react with the peroxidase-conjugated secondary antibody. After a 20 minute incubation at room temperature, stop solution containing 1N hydrochloric acid was layered on top of each well, and the plates were read at 450 nanometers. Titers were determined as the highest dilution with an absorbance value 3 times greater than the average absorbance of the negative control.

3.10: Neutralizing Antibody Assay

To measure the functionality of antibodies against the drift variant ma2009 H1N1 IAV virus, neutralizing antibody assays were performed on pre-challenge serum samples that were collected 21 days post vaccination 2 (Fink et al. 2018). Before running the assay, 96-well plates were seeded with MDCK cells. When the plates were 100 percent or over confluent, the assay was started.

Infectious media containing Dulbecco's Modified Eagle's Medium (DMEM), BSA, and N-acetyl trypsin was made. An aliquot of the infectious media was used to create the viral media, which contained the drift variant ma2009 H1N1 IAV virus. Heat-inactivated serum samples were serially diluted in infectious media, and then viral media was layered on top; the samples were incubated at room temperature for 1 hour. During this time, the TCID50 back-titer plate was prepared by serially diluting the viral media in infectious media. The MDCK cell media was removed from the plates, followed by washes with PBS containing calcium and magnesium. The serum sample dilutions suspended in viral media were then plated in replicates of 4 and incubated for 24 hours at 32C.

Following the incubation, the media was removed and the plates were washed with PBS containing calcium and magnesium. New infectious media was added, and the plates were incubated for 6 days at 32C.

After the 6 day incubation, the media was dumped out and the plates were fixed with zinc formalin. The plates were then stained with naphthol blue black. After washing with deionized water, the neutralizing antibody titer was calculated as the highest dilution that did not show cytopathic effects (CPE) in 50 percent of the wells.

3.11: Challenge with the Drift Variant Influenza A Virus

At 8 weeks post birth and 3 week following vaccination 2, the offspring were challenged with the drift variant ma2009 H1N1 IAV containing a K166Q mutation on the hemagglutinin glycoprotein; challenging with the drift variant mimics the seasonal discrepancy between IAV vaccine strains and circulating IAV infectious strains in human populations (Fink et al. 2018. Linderman et al. 2014; Castelan-Vega et al. 2014; Yasuhara et al. 2017). On the day of the challenge, body weight and temperature were recorded as a baseline measurement. The infection solution was made by diluting the drift variant viral stock generated by the lab of Dr. Sabra Klein (Johns Hopkins Bloomberg School of Public Health) in sterile DMEM to reach a lethal tissue culture infectious dose 50 (TCID50) of 10^5. Two aliquots of the infection solution were placed on ice, while the third aliquot was preserved at -80C for future titering.

The offspring were anesthetized with a sub-lethal dose of a ketamine/xylazine mixture by intraperitoneal injection. The dose used for

anesthetizing was determined by body weight; mice under 20 grams received 75 μL of the drug cocktail while mice over 20 grams were given 100 μL of the drug cocktail. After ensuring the animals were completely anesthetized, they were placed on a heating pad to prevent heat loss, and lubricant was added to the eyes to prevent drying. The offspring were then intranasally inoculated by administering 30 μL of the infection solution into the nasal cavity. The infection solution was slowly added drop-wise, allowing the mouse to inhale each drop and alternating nostrils to ensure even distribution of the virus into both lungs.

From the 1st wave of mating, 2 male and 2 female offspring from each treatment group acted as unchallenged controls; these mice were sham infected by intranasally inoculating with 30 μL of sterile PBS. In addition, the unvaccinated controls from the 2nd wave of mating were sham infected with the same volume of PBS.

Following challenge with the drift variant ma2009 H1N1 IAV, body weight and temperature were measured daily. As stated in the protocol approved by the Animal Care and Use Committee, if during the disease course the offspring lost more than 35 percent of their initial body weight or 10 percent of their initial temperature, they were euthanized. In addition, morbidity following challenge was measured by the presentation of clinical signs including piloerection, hunched posture, lack of an escape response, and dyspnea (Foltz and Ullman-Cullere 1999; Burkholder et al. 2012).

3.12: Animal Euthanasia and Tissue Collection

At different points during the disease course (1, 3, 5, and 28 days post challenge), the offspring were euthanized and tissue samples were collected. Animals were euthanized by the inhalation of isoflurane and subsequent rupture of the diaphragm.

Blood, lungs, spleen, thymus, and mediastinal lymph nodes were collected from the animals. Blood was collected through the inferior vena cava, and then spun down at 10,000 x gravity for 10 minutes to obtain serum. The entire left lung of the animal was homogenized using tissue dissociator C-tubes (Miltenyi Biotec) and preserved in cryopreservation media containing fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO). The superior, middle, and inferior lobes of the right lung were snap frozen for prospective TCID50 assays to measure viral concentration. The post-caval lobe of the right lung was placed in RNAprotect (Qiagen) for future work on gene and protein expression. The spleen, thymus, and mediastinal lymph nodes were homogenized using frosted microscope slides and preserved in cryopreservation media for immune cell analysis by flow cytometry.

3.13: Statistical Analysis

Differences in birth outcomes including reproductive success, litter size, and proportion of males to females between treatment groups were analyzed using Student's t-test. Data collected from the gestation day 17

euthanasia was also analyzed using Student's t-test. Results such as pre-challenge IgG and neutralizing antibody titers were graphed using Prism (GraphPad) and compared using two-way ANOVAs with multiple comparisons. Body weight and temperature change following challenge was analyzed and compared using non-linear regression. Differences were considered statistically significant if the $P\text{-}value < 0.05$.

PART 4: RESULTS

4.1: Reproductive Success and Birth Outcomes

4.1.1: Reproductive Success

Pregnancy was confirmed by a weight gain of more than 2 grams by gestation day 10 (Heyne et al. 2015; Mader et al. 2009; Pauley and Washington 2017). From the 1st wave of mating, there were 3 pregnancies from the 0 ppb treatment group and 3 pregnancies from the 100 ppb treatment group (Table 2). From the 2nd wave of mating, there were 6 pregnancies from the 0 ppb treatment group and 4 pregnancies from the 100 ppb treatment group (Table 3). By pooling the data from the 1st and 2nd waves, the percent of successful pregnancies for the 0 ppb group was 47.4 percent, while the percent of successful pregnancies for the 100 ppb group was 36.8 percent (Table 6); the difference in number of successful pregnancies was not statistically significant between the two treatment groups (P=0.52, 95% confidence interval=-0.44 to 0.23). In addition, all litters from both waves of mating were delivered on gestation day 19, which is within the range of expected gestation length (Murray et al. 2010; McCarthy et al. 2018).

Table 2: Reproductive success for the 1st wave of mating; no significance.

Treatment	# of Animals Mated	# of Successful Pregnancies	% of Successful Pregnancies
0 ppb	19	3	15.8%
100 ppb	19	3	15.8%

Table 3: Reproductive success for the 2nd wave of mating; no significance.

Treatment	# of Animals Mated	# of Successful Pregnancies	% of Successful Pregnancies
0 ppb	16	6	37.5%
100 ppb	16	4	25%

4.1.2: Litter Size

For the 1st wave of mating, litter size from the 0 ppb treatment group ranged from 4 to 10 pups, with an average of 7 pups per litter; litters from the 100 ppb treatment group ranged from 7 to 8 pups, with an average of 7.3 pups per litter (Table 4). For the 2nd wave of mating, litter size from the 0 ppb group ranged from 2 to 9 pups, with an average of 5.7 pups per litter, while litters from the 100 ppb group ranged from 4 to 10 pups, with an average of 7.5 pups per litter (Table 5). By pooling the data from the 1st and 2nd waves, the average litter size for the 0 ppb treatment group was 6.1 pups and the average litter size for the 100 ppb treatment group was 7.4 pups (Table 6); the difference in litter size between the two treatment groups was not statistically significant (P=0.28, 95% confidence interval=-1.21 to 3.85).

From the 2nd wave of mating, 2 pups from the 0 ppb group and 1 pup from the 100 ppb group were lost to cannibalism during the first week following birth. This was perhaps due to stress, caused by excessive noise and vibration, following delivery of the pups (Lane-Petter 1968; Poley 1974). In addition, pup cannibalism is more common in nulliparous females (Weber, Olsson, and Algers 2007; Weber et al. 2013).

4.1.3: Proportion of Males to Females

One week following birth, pups were sexed. For the 0 ppb group from the 1st wave of mating, the number of males in each litter ranged from 3 to 8, while the number of females in each litter ranged from 1 to 4. For the 100 ppb group from the 1st wave of mating, the number of males per litter ranged from 3 to 6 and the number of females per litter ranged from 2 to 4. Overall from the 1st wave of mating, there were 14 male pups and 7 female pups from the 0 ppb treatment group, and 12 male pups and 10 female pups from the 100 ppb treatment group (Table 4).

From the 2nd wave of mating, the number of males per litter ranged from 2 to 5, while the number of females per litter ranged from 1 to 4 for the 0 ppb treatment group. For the 100 ppb treatment group from the 2nd wave of mating, the number of males in each litter ranged from 1 to 6, and the number of females in each litter ranged from 2 to 5. Overall from the 2nd wave of mating, there were 19 male pups and 13 female pups from the 0 ppb group, and 17 male pups and 12 female pups from the 100 ppb group (Table 5).

By pooling the data from the 1st and 2nd waves of mating for the 0 ppb treatment group, the total number of male pups was 33 and the total number of female pups was 20, a difference that was statistically significant (P=0.05, 95% confidence interval=-3.27 to 0.02). However, by pooling the data for the 100 ppb treatment group, the total number of male pups was 29 and the total

number of female pups was 22 (Table 6); this difference was not statistically significant (*P*=0.26, 95% confidence interval=-2.83 to 0.83).

Table 4: Litter composition for the 1st wave of mating; no significance.

Treatment	Number of Litters	Average Litter Size	Total # of Male Pups	Range for Males per Litter	Total # of Female Pups	Range for Females per Litter
0 ppb	3	7	14	5	7	3
100 ppb	3	7.3	12	3	10	2

Table 5: Litter composition for the 2nd wave of mating; no significance.

Treatment	Number of Litters	Average Litter Size	Total # of Male Pups	Range for Males per Litter	Total # of Female Pups	Range for Females per Litter
0 ppb	6	5.7	19	3	13	3
100 ppb	4	7.5	17	5	12	3

Table 6: Birth outcomes data pooled from the 1st and 2nd waves of mating; no significance.

Treatment	% of Successful Pregnancies	Average Litter Size	Total # of Male Pups	Total # of Female Pups
0 ppb	47.4%	6.1	33	20
100 ppb	36.8%	7.4	29	22

4.1.4: Weight and Length of Pups at 1 Week of Age

One week following birth, the offspring were measured for weight and crown-to-rump length. For male pups from the 1st wave of mating, the average weight was 3.31 grams and the average length was 35.24 centimeters for the 0 ppb treatment group. For male pups from the 100 ppb

treatment group, the average weight was 3.09 grams and the average length was 33.83 centimeters (Table 7). The difference in both weight and length of male pups between treatment groups was statistically significant (*P*=0.05, 95% confidence interval=-0.43 to 0.00 and *P*=0.05, 95% confidence interval=-2.81 to -0.01 respectively). For female pups from the 1st wave of mating, the average weight was 3.01 grams and the average length was 33.69 centimeters for the 0 ppb treatment group. For female pups from the 100 ppb treatment group, the average weight was 3.03 grams and the average length was 32.60 centimeters (Table 7). The difference in both weight and length of female pups between treatment groups was not significant (*P*=0.87, 95% confidence interval=-0.28 to 0.32 and *P*=0.09, 95% confidence interval=-2.38 to 0.21 respectively).

From the 2nd wave of mating, the average weight was 3.13 grams and the average length was 35.37 centimeters for male pups from the 0 ppb group. The average weight was 3.30 grams and the average length was 34.73 centimeters for male pups from the 100 ppb group (Table 8). The difference in both weight and length of male pups between treatment groups was not significant (*P*=0.99, 95% confidence interval=-0.23 to 0.23 and *P*=0.25, 95% confidence interval=-1.75 to 0.48 respectively). For female pups from the 2nd wave of mating, the average weight was 3.24 grams and the average length was 34.82 centimeters for the 0 ppb treatment group, while the average weight was 3.19 grams and the average length was 34.88 centimeters for the

100 ppb treatment group (Table 8). The difference in both weight and length of female pups between treatment groups was not significant (P=0.71, 95% confidence interval=-0.32 to 0.22 and P=0.93, 95% confidence interval=-1.36 to 1.48 respectively).

By pooling the data for male pups in the 0 ppb treatment group, the average weight was 3.30 grams and the average length was 35.31 centimeters, while the average weight was 3.21 grams and the average length was 34.36 centimeters for male pups from the 100 ppb treatment group (Table 9). The difference in weight for male pups between treatment groups was not significant (P=0.26, 95% confidence interval=-0.25 to 0.07); however, the difference in crown-to-rump length was significant (P=0.03, 95% confidence interval=-1.81 to -0.10). By pooling the data for female pups in the control group, the average weight was 3.16 grams and the average length was 34.42 centimeters, while the average weight was 3.12 grams and the average length was 33.84 centimeters for female pups from the arsenic group (Table 9). Although the average weight and average length for the experimental group were lower compared to the control group, the difference was not significant (P=0.68, 95% confidence interval=-0.24 to 0.16 and P=0.30, 95% confidence interval=-1.68 to 0.52 respectively).

Table 7: Weight and crown-to-rump length averages at 1 week of age for the 1st wave of mating, stratified by sex. N=7-14 pups from 3 litters; $P < 0.10$ indicated by † and statistical significance indicated by *$P < 0.05$.

Treatment	Average Weight of Male Pups (g)	Average Length of Male Pups (cm)	Average Weight of Female Pups (g)	Average Length of Female Pups (cm)
0 ppb	3.31	35.24	3.01	33.69
100 ppb	3.09*	33.83*	3.03	32.60†

Table 8: Weight and crown-to-rump length averages at 1 week of age for the 2nd wave of mating, stratified by sex. N=12-19 pups from 4 to 5 litters; no significance.

Treatment	Average Weight of Male Pups (g)	Average Length of Male Pups (cm)	Average Weight of Female Pups (g)	Average Length of Female Pups (cm)
0 ppb	3.13	35.37	3.24	34.82
100 ppb	3.30	34.73	3.19	34.88

Table 9: Pooled weight and crown-to-rump length averages at 1 week of age for the 1st and 2nd waves of mating, stratified by sex. N=20-33 pups from 7 to 8 litters; statistical significance indicated by *$P < 0.05$.

Treatment	Average Weight of Male Pups (g)	Average Length of Male Pups (cm)	Average Weight of Female Pups (g)	Average Length of Female Pups (cm)
0 ppb	3.30	35.31	3.16	34.42
100 ppb	3.21	34.36*	3.12	33.84

4.2: Gestation Day 17 Euthanasia

There were 10 females from the 0 ppb treatment group that did not get pregnant during the 1st or 2nd waves of mating. In preparation for the gestation day 17 euthanasia, 5 females were exposed to 0 ppb treated water and 5 females were exposed to 100 ppb treated water for 2 weeks prior to mating. After mating, 2 females from the 0 ppb group and 1 female from the 100 ppb group were confirmed to be pregnant.

4.2.1: Litter Size and Fetal Resorptions

The first litter from the control group had a total of 2 fetuses with 1 fetal resorption, indicated by necrotic placental tissue in the uterine horn (Ward, Elmore, and Foley 2012; Flores et al. 2014; Miner et al. 2016). The second litter from the control group had a total of 7 fetuses with 1 fetal resorption. The litter from the experimental group had a total of 7 fetuses with no fetal resorptions.

4.2.2: Weight and Length of Fetuses

Fetuses from the 0 ppb treatment group had an average weight of 0.89 grams and an average crown-to-rump length of 19.63 centimeters. Overall, fetuses from the 100 ppb treatment group had lower body measurements, with an average weight of 0.77 grams and an average crown-to-rump length of 17.36 centimeters (Table 10). The difference in fetal weight was significant

between the two treatment groups (P=0.05, 95% confidence interval=-0.23 to 0.00); however, the difference in fetal length was not (P=0.26, 95% confidence interval=-6.55 to 2.01). Because the fetuses were not to term, sex could not be visually determined; future work will be directed towards identifying the sex of the fetuses to stratify the weight and crown-to-rump length data.

4.2.3: Fetal Tissue Weight

The average placenta weight was higher for the 100 ppb group; the average weight of the fetal tissue was 0.082 grams for the control group and 0.084 grams for the experimental group. On the contrary, the 100 ppb treatment group had lower average lung and heart weights compared to the 0 ppb treatment group. The average weight of the lungs was 0.0318 grams for the control group and 0.0254 grams for the experimental group, while the average heart weight was 0.0047 grams for the control group and 0.0038 grams for the experimental group (Table 10). Overall the difference in placenta, lung, and heart tissue weight between the two groups was not significant (P=0.85, 95% confidence interval=-0.02 to 0.02, P=0.20, 95% confidence interval=-0.02 to 0.00, and P=0.18, 95% confidence interval=-0.01 to 0.00 respectively).

Table 10: Gestation day 17 euthanasia measurements. N=3-7 fetuses from 1 to 2 litters; statistical significance indicated by *$P < 0.05$.

Treatment	Average Weight (g)	Average Length (cm)	Average Placenta Weight (g)	Average Lung Weight (g)	Average Heart Weight (g)
0 ppb	0.89	19.63	0.082	0.0318	0.0047
100 ppb	0.77*	17.36	0.084	0.0254	0.0038

4.3: Pre-Challenge Influenza A Virus Vaccine Immunogenicity

4.3.1: Anti-Influenza A Virus Immunoglobulin G Titers

Immunoglobulin G is the most abundant antibody in circulation and tissue. IgG mediates protection against local and systemic antigens by neutralizing, opsonizing, and eliminating pathogens such as viruses and bacteria (Vidarsson, Dekkers, and Rispens 2014). Vaccines provide protection by inducing the production of antigen-specific IgG antibodies that can go into action upon reintroduction of the pathogen (Wang, Bournazos, and Ravetch 2018).

Before challenge with the drift variant ma2009 H1N1 IAV, arsenic-exposed female offspring from the 1st wave of mating had significantly higher total anti-ma2009 IAV IgG titers compared to arsenic-exposed male offspring (P=0.04, 95% confidence interval=-95.54 to -1.33). In addition, even though 100 ppb females had higher total anti-ma2009 IAV IgG titers compared to 0 ppb females, the difference was not statistically significant (P=0.11, 95% confidence interval=-101.50 to 6.90); the difference in total anti-ma2009 IAV IgG titers between male offspring from the control group and male offspring

from the experimental group was also not significant (Figure 4; P=0.97, 95% confidence interval=-50.16 to 36.40 respectively). When graphed by litter average, there were no significant differences in anti-ma2009 IAV IgG titers between treatment groups or sexes (Supplemental Figure 1).

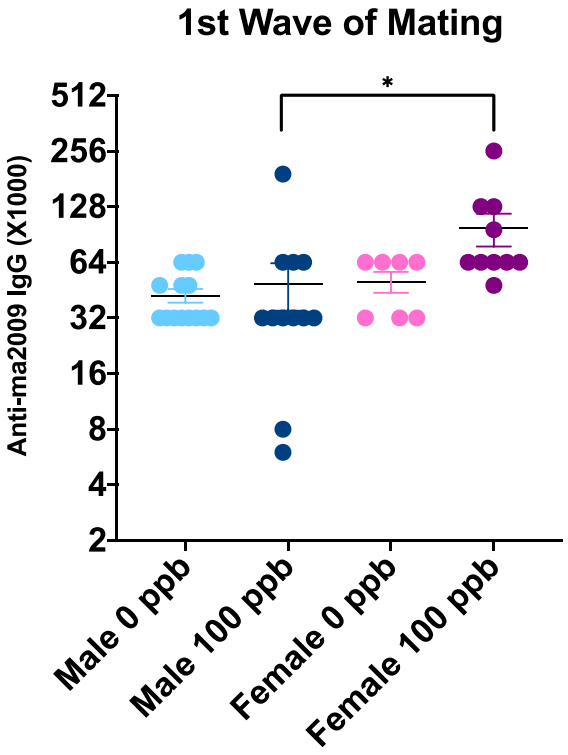

Figure 4: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG titers for the 1st wave of mating. N=7-14 offspring from 3 litters; statistical significance indicated by *$P < 0.05$.

From the 2nd wave of mating, female offspring from the 0 ppb treatment group had higher total anti-ma2009 IAV IgG titers compared to male offspring from the 0 ppb treatment group; however, this difference was

not significant (P=0.07, 95% confidence interval=-40.78 to 1.25). The difference in anti-ma2009 IAV IgG titers between 0 ppb males and 100 ppb males, as well as between 0 ppb females and 100 ppb females was also not significant (Figure 5; P=0.96, 95% confidence interval=-23.22 to 15.98 and P=0.94, 95% confidence interval=-19.07 to 29.74 respectively). Furthermore, there were no significant differences in anti-ma2009 IAV IgG titers between treatment groups or sexes when graphed by litter average (Supplemental Figure 2).

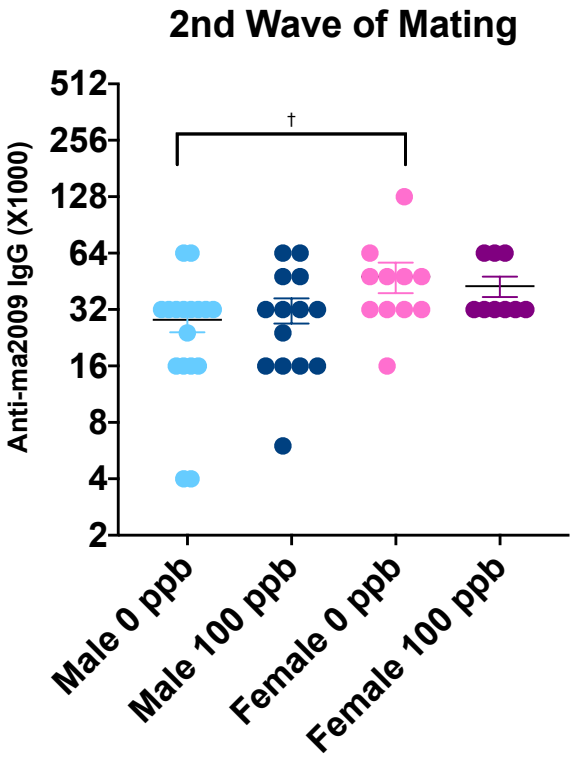

Figure 5: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG titers for the 2nd wave of mating. N=9-17 offspring from 4 to 5 litters; $P <$ 0.10 indicated by †.

4.3.2: Anti-Influenza A Virus Immunoglobulin G1 to G2a/c Titers

There are four subclasses of IgG. Human IgG1, which is equivalent to mouse IgG2a, is the most abundant IgG subclass; in the C57BL/6 mouse strain, IgG2a is indistinguishable from IgG2c and are referred to in parallel (Zhang, Goldschmidt, and Salter 2012; Dekkers et al. 2017). Human IgG1 and mouse IgG2a/c play an important role in providing protection against protein antigens such as viruses. Human IgG4, which is equivalent to mouse IgG1, is one of the less abundant IgG subclasses, but plays a key role in neutralizing polysaccharide antigens associated with bacterial infections (Vidarsson, Dekkers, and Rispens 2014). In this setting, a smaller IgG1 to IgG2a/c ratio indicates greater protection from the vaccine.

For vaccinated offspring from the 1st wave of mating, females from the control group had significantly higher IgG1 to IgG2a/c titer ratios compared to males from the control group (P=0.01, 95% confidence interval=-1.35 to -0.13). However, there was no significance difference in IgG1 to IgG2a/c titer ratios when comparing between the two treatment groups for both male and female offspring (Figure 6; P=0.99, 95% confidence interval=-0.53 to 0.51 and P=0.65, 95% confidence interval=-0.37 to 0.93 respectively). When graphed by litter average, there were no statistically significant differences in anti-ma2009 IAV IgG1 to IgG2a/c titer ratios between treatment groups or sexes (Supplemental Figure 3).

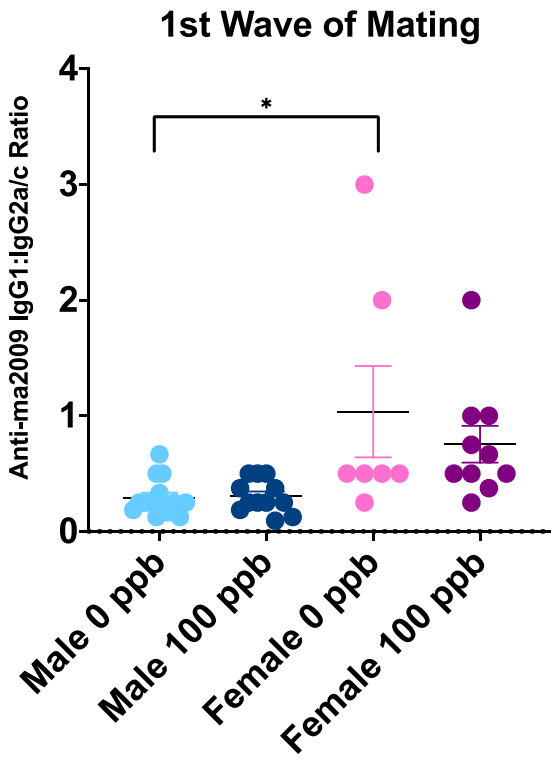

Figure 6: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG1 to IgG2a/c titer ratios for the 1st wave of mating. N=7-14 offspring from 3 litters; statistical significance indicated by *$P < 0.05$.

For vaccinated offspring from the 2nd wave of mating, there was no statistical significance in IgG1 to IgG2a/c titer ratios between the four different groups. Additionally, the IgG1 to IgG2a/c titer ratios did not differ when comparing between the two treatment groups for either male or female offspring (Figure 7; P=0.59, 95% confidence interval=-0.69 to 0.25 and P=0.99, 95% confidence interval=-0.58 to 0.58 respectively). There were no significant differences in anti-ma2009 IAV IgG1 to IgG2a/c titer ratios

between either treatment groups or sexes when graphed by litter average (Supplemental Figure 4).

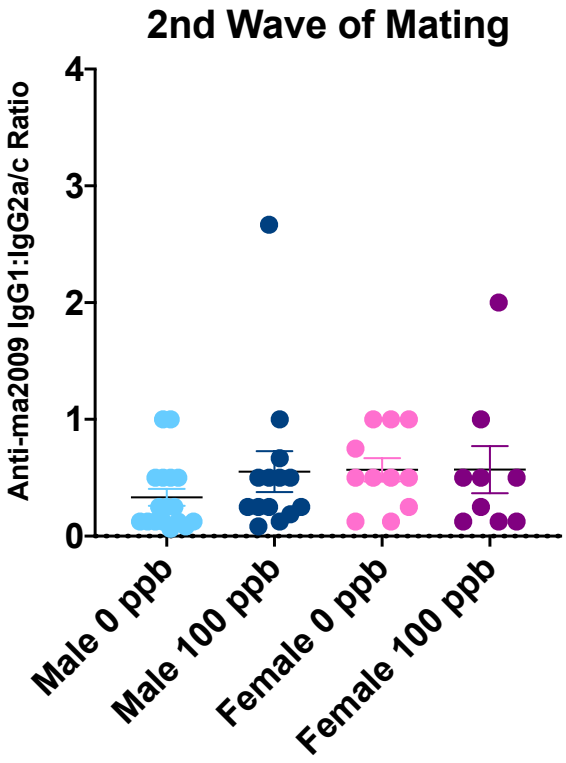

Figure 7: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG1 to IgG2a/c titer ratios for the 2nd wave of mating. N=9-17 offspring from 4 to 5 litters; no significance.

4.3.3: Antibody Neutralization Capacity

From the 1st wave of mating, female offspring from the 100 ppb group had significantly higher neutralizing antibody titers against the drift variant ma2009 H1N1 IAV compared to both 100 ppb male offspring and 0 ppb female offspring (*P*=0.02, 95% confidence interval=-7963 to -705.7 and

P=0.01, 95% confidence interval=-9458 to -1105 respectively). Arsenic did not have an effect on antibody neutralization capacity for male offspring (Figure 8; *P*=0.99, 95% confidence interval=-3123 to 3545). If graphed by litter average, the statistically significant difference in neutralizing antibody titers between treatment groups or sexes recedes (Supplemental Figure 13).

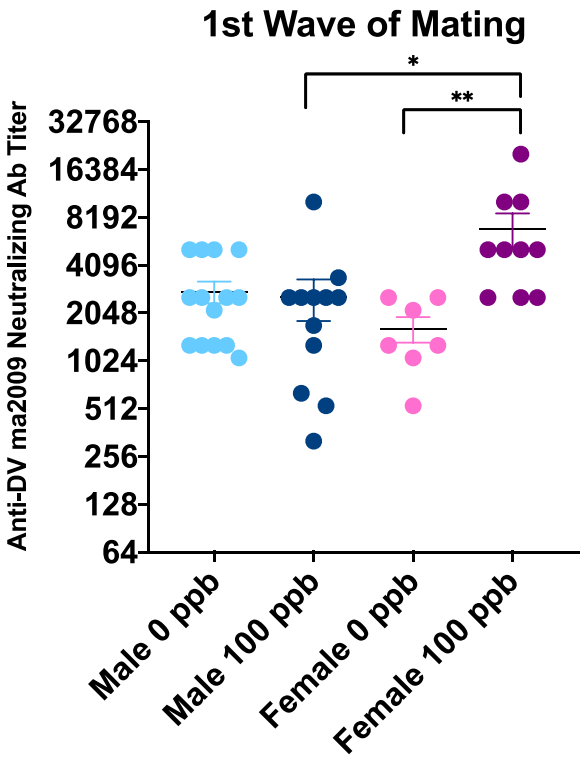

Figure 8: Pre-challenge (21 days post vaccination 2) anti-DV ma2009 IAV neutralizing antibody titers for the 1st wave of mating. N=7-14 offspring from 3 litters; statistical significance indicated by $*P < 0.05$ and $**P < 0.01$.

From the 2nd wave of mating, there was no statistically significant difference in neutralizing antibody titers against the drift variant ma2009

H1N1 IAV between the four different groups. In addition, there was no difference in neutralizing antibody titers between treatment groups for both male and female offspring (Figure 9; *P*=0.52, 95% confidence interval=-1359 to 4236 and *P*=0.98, 95% confidence interval=-2908 to 3964 respectively). When graphed by litter average, there were no significant differences in neutralizing antibody titers between treatment groups or sexes (Supplemental Figure 14).

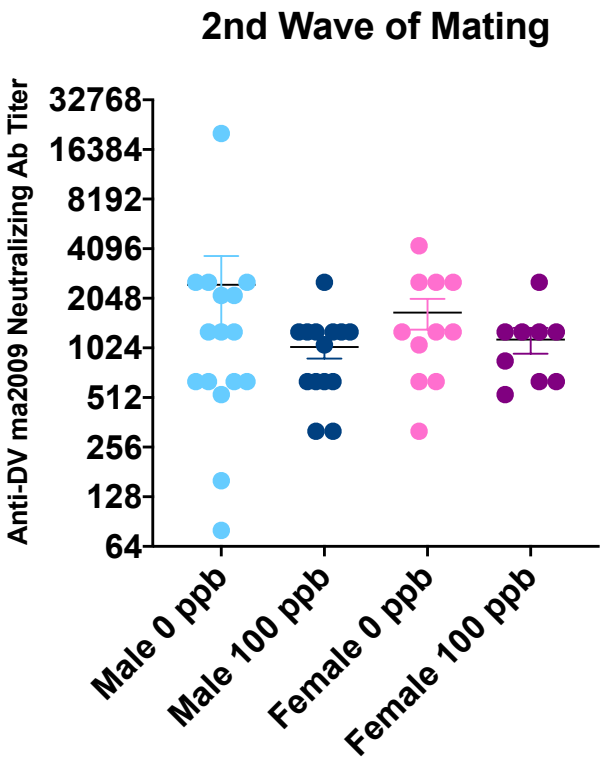

Figure 9: Pre-challenge (21 days post vaccination 2) anti-DV ma2009 IAV neutralizing antibody titers for the 2nd wave of mating. N=9-17 offspring from 4 to 5 litters; no significance.

4.4: Phenotypic Measurements Following Challenge

4.4.1: Body Weight Change

Following challenge with the drift variant ma2009 H1N1 IAV, 0 ppb male offspring from the 1st wave of mating had the greatest percent change in body weight compared to all other offspring groups (Figure 10). Furthermore, male offspring from the 0 ppb treatment group had a significantly greater weight gain progression following challenge compared to male offspring from the 100 ppb treatment group (Figure 11; P=0.001). In addition, 0 ppb females had a greater weight gain progression following challenge compared to 100 ppb females, although this difference was not statistically significant (Figure 12; P=0.09). When graphed by litter average, the statistical significance between treatment groups was sustained (Supplemental Figures 15, 16, and 17).

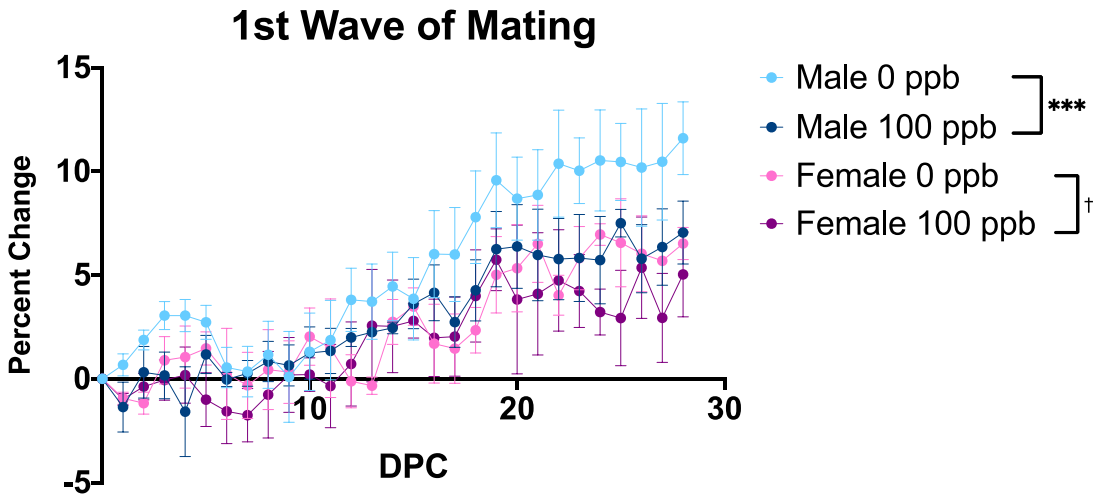

Figure 10: Percent body weight change days post challenge (DPC) for offspring from the 1st wave of mating. N=5-12 offspring from 3 litters; $P < 0.10$ indicated by † and statistical significance indicated by ***$P < 0.001$.

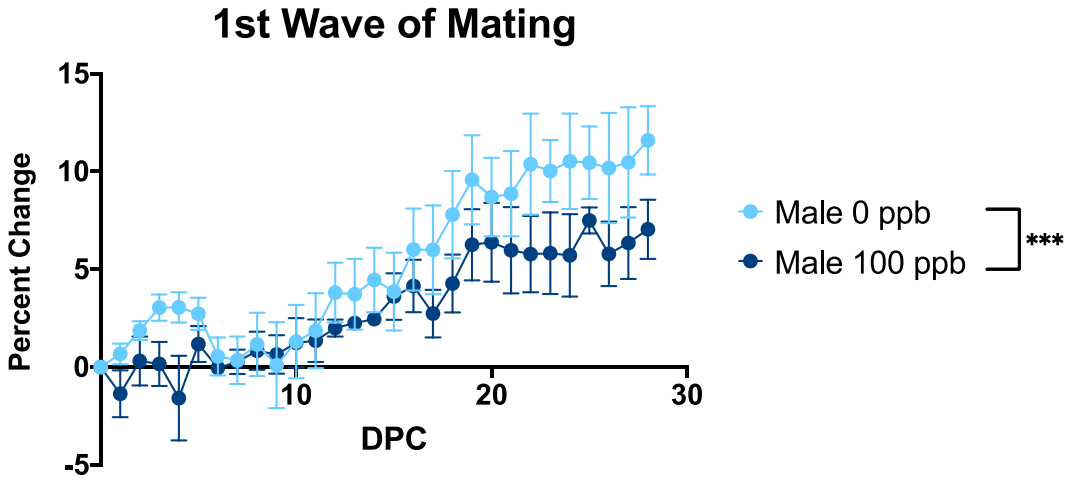

Figure 11: Percent body weight change days post challenge (DPC) for male offspring from the 1st wave of mating. N=10-12 offspring from 3 litters; statistical significance indicated by ***$P < 0.001$.

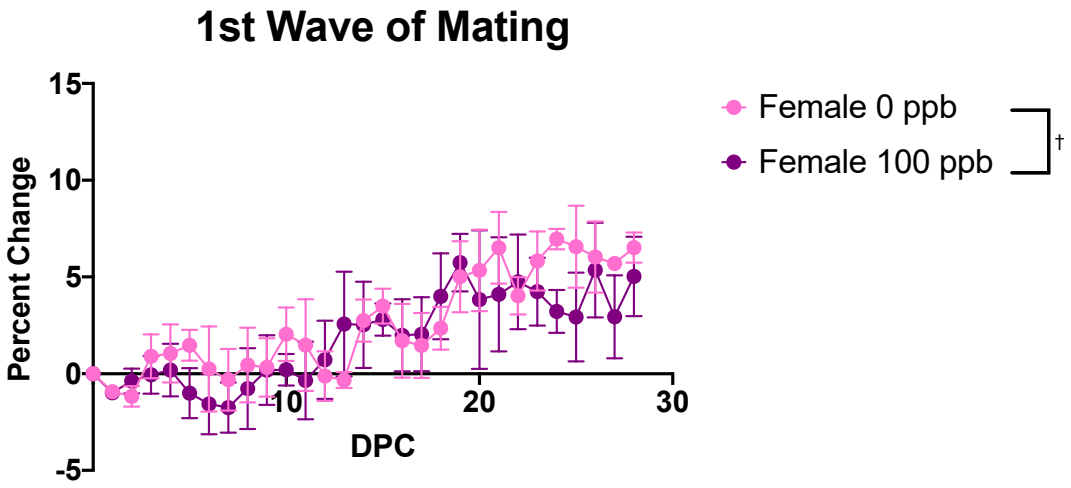

Figure 12: Percent body weight change days post challenge (DPC) for female offspring from the 1st wave of mating. N=5-8 offspring from 3 litters; $P < 0.10$ indicated by †.

From the 2nd wave of mating, female offspring from the experimental treatment group had the greatest loss in weight following challenge (Figure 13). Overall, both male and female offspring from the 0 ppb treatment group differed significantly in body weight change following challenge compared to the 100 ppb treatment group, with vaccinated offspring from the control group experiencing less weight loss after challenge with the drift variant ma2009 H1N1 IAV (Figure 14 and 15; P=0.002 and P=0.02 respectively). When graphed by litter average, differences in weight change following challenge remained statistically significant between the 0 ppb females and the 100 ppb females (Supplemental Figures 18, 19, and 20).

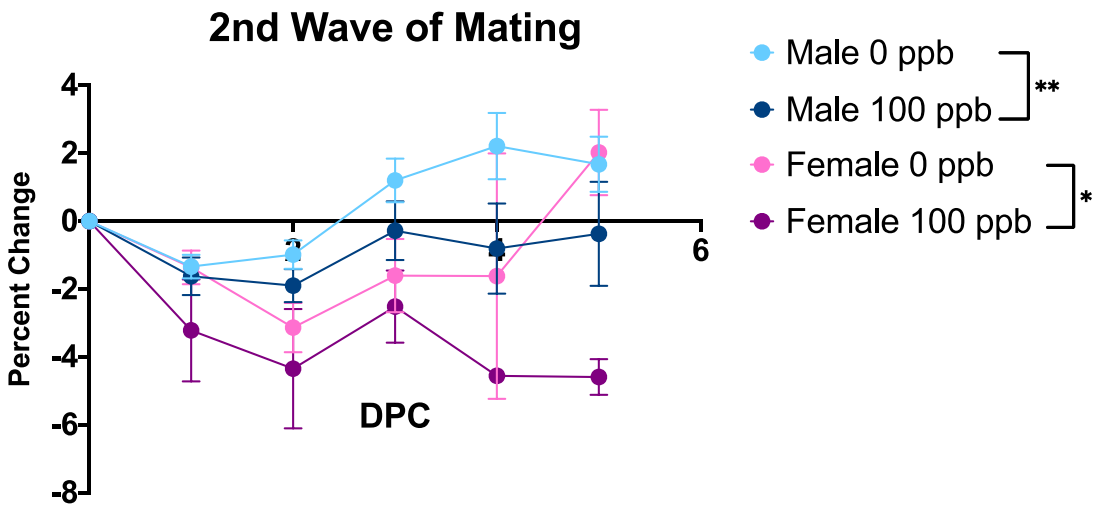

Figure 13: Percent body weight change days post challenge (DPC) for offspring from the 2nd wave of mating. N=9-17 offspring from 4 to 5 litters; statistical significance indicated by *$P < 0.05$ and **$P < 0.01$.

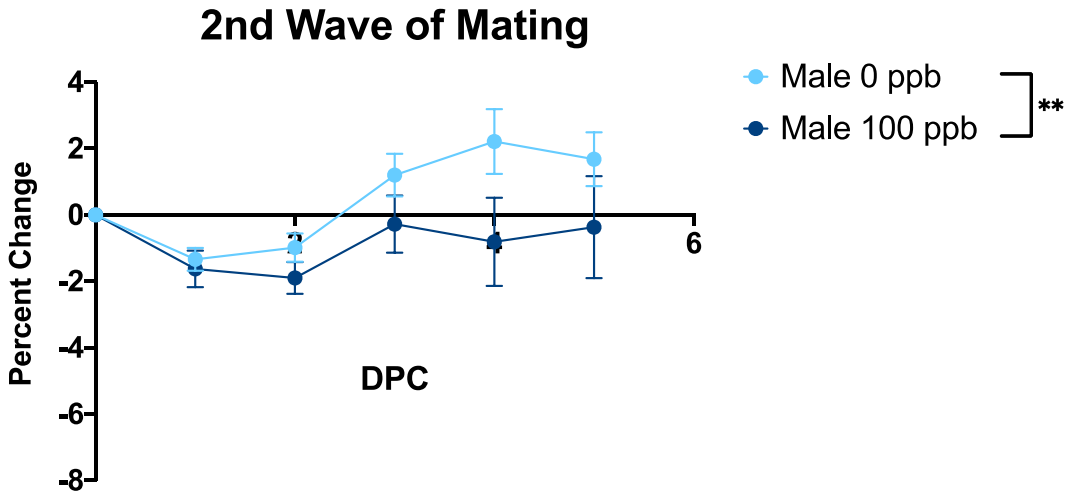

Figure 14: Percent body weight change days post challenge (DPC) for male offspring from the 2nd wave of mating. N=14-17 offspring from 4 to 5 litters; statistical significance indicated by **$P < 0.01$.

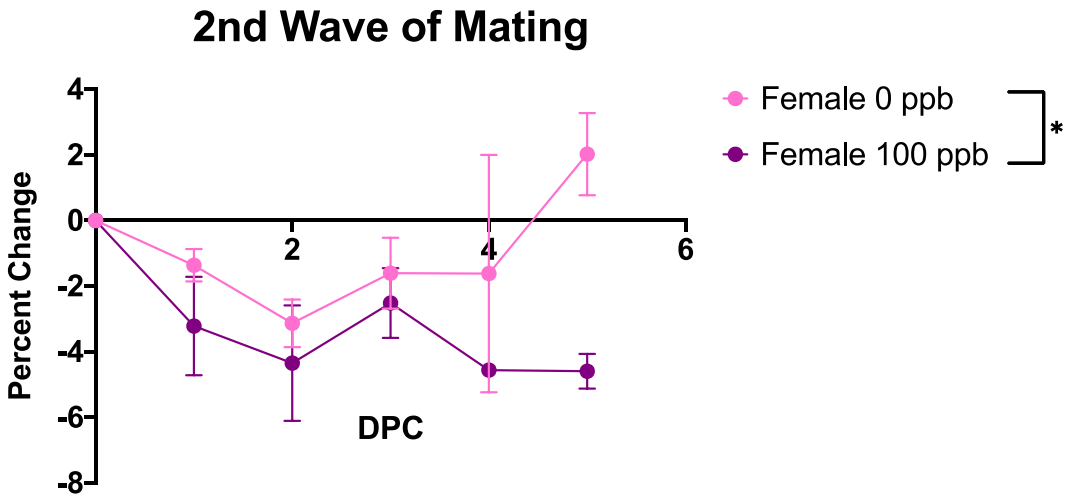

Figure 15: Percent body weight change days post challenge (DPC) for female offspring from the 2nd wave of mating. N=9-11 offspring from 4 to 5 litters; statistical significance indicated by *$P < 0.05$.

4.4.2: Temperature Change

Following challenge with the drift variant ma2009 H1N1 IAV, vaccinated offspring from the 1st wave of mating did not experience significant changes in temperature (Figure 16). For both male and female offspring, *in utero* arsenic exposure did not have an effect on temperature change after challenge (Figure 17 and 18; P=0.89 and P=0.32 respectively). When graphed by litter average, there was no significant difference in temperature change following challenge between treatment groups (Supplemental Figures 21, 22, and 23).

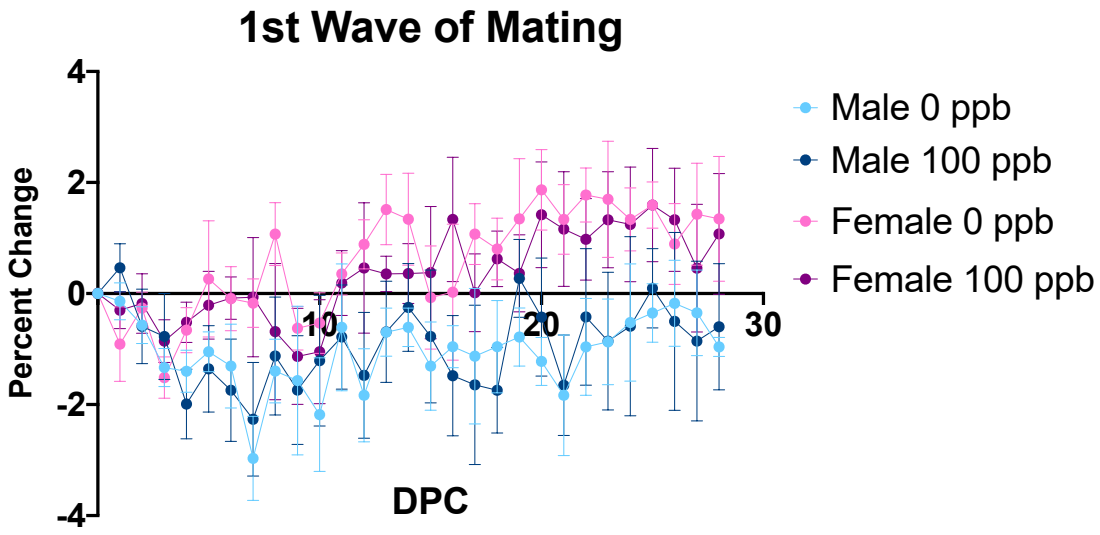

Figure 16: Percent temperature change days post challenge (DPC) for offspring from the 1st wave of mating. N=5-12 offspring from 3 litters; no significance.

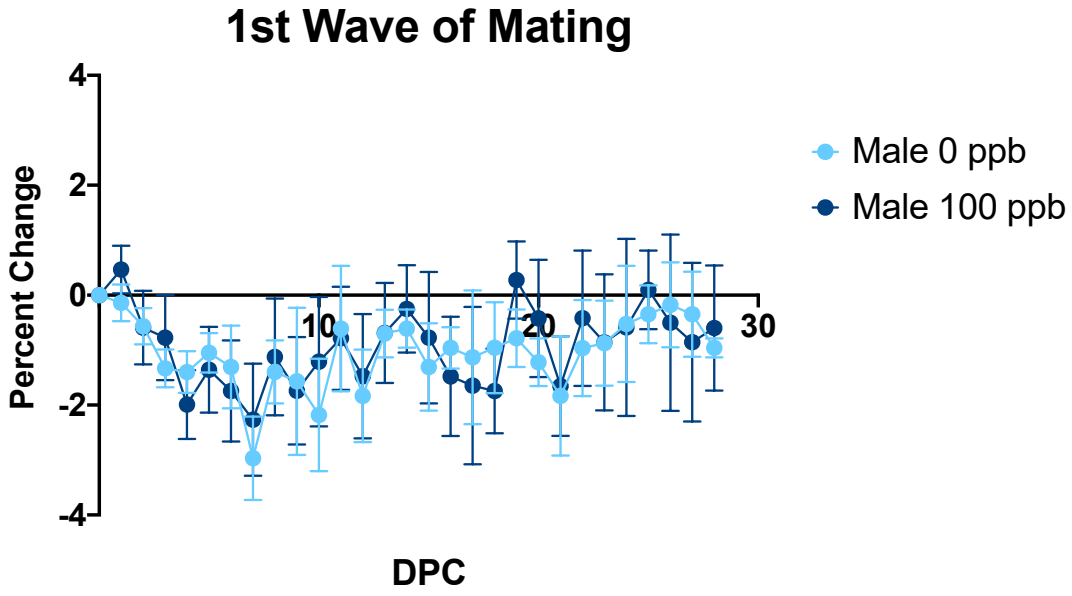

Figure 17: Percent temperature change days post challenge (DPC) for male offspring from the 1st wave of mating. N=10-12 offspring from 3 litters; no significance.

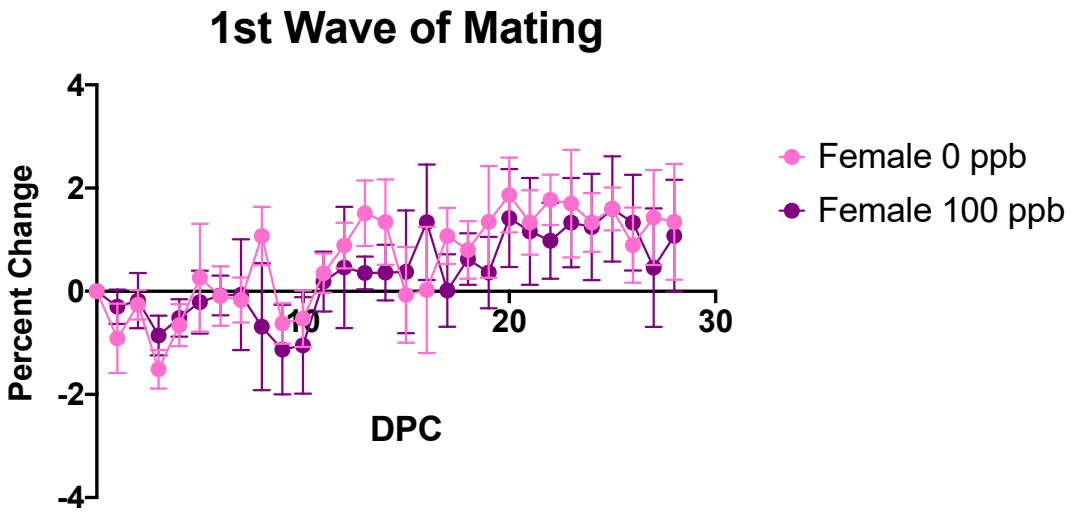

Figure 18: Percent temperature change days post challenge (DPC) for female offspring from the 1st wave of mating. N=5-8 offspring from 3 litters; no significance.

For vaccinated offspring from the 2nd wave of mating, males and females from both the control and experimental groups did not experience critical changes in temperature following challenge (Figure 19). The difference in temperature between 0 ppb males and 100 ppb males was not significant; however, the drop in temperature for 100 ppb females was statistically significant when compared to 0 ppb females (Figure 20 and 21; P=0.31 and P=0.001 respectively). When graphed by litter average, the difference in temperature change following challenge remained statistically significant between the 0 ppb females and the 100 ppb females (Supplemental Figures 24, 25, and 26).

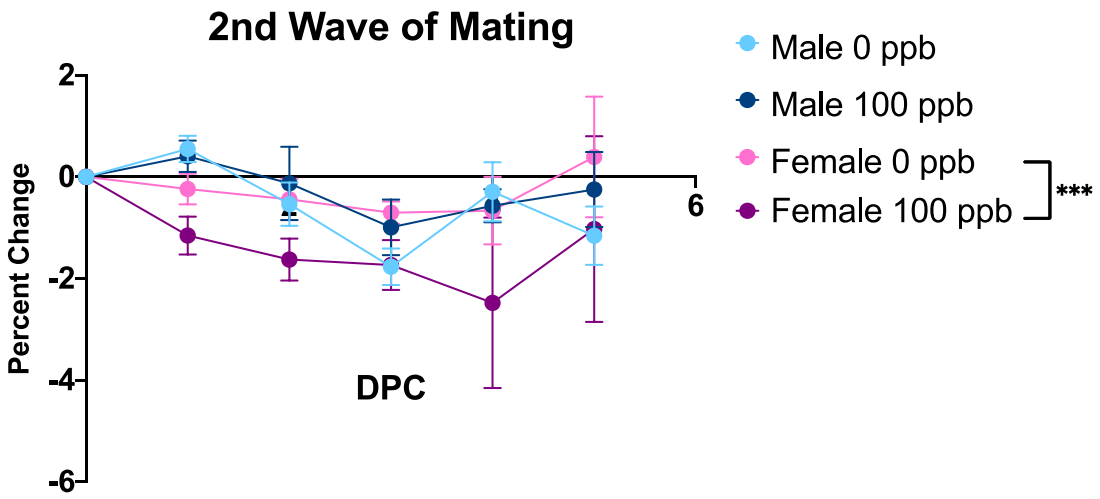

Figure 19: Percent temperature change days post challenge (DPC) for offspring from the 2nd wave of mating. N=9-17 offspring from 4 to 5 litters; statistical significance indicated by ***$P < 0.001$.

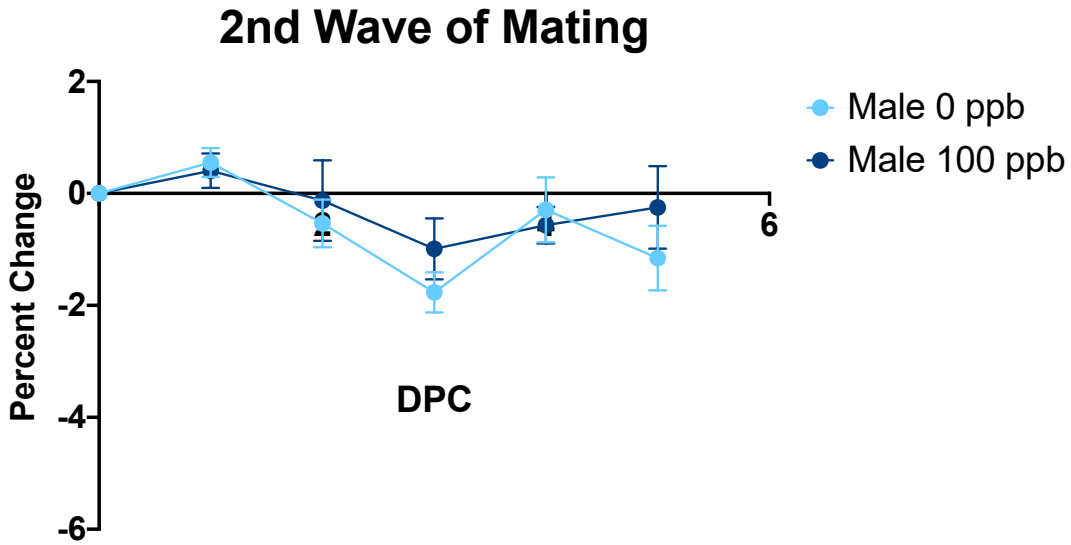

Figure 20: Percent temperature change days post challenge (DPC) for male offspring from the 2nd wave of mating. N=14-17 offspring from 4 to 5 litters; no significance.

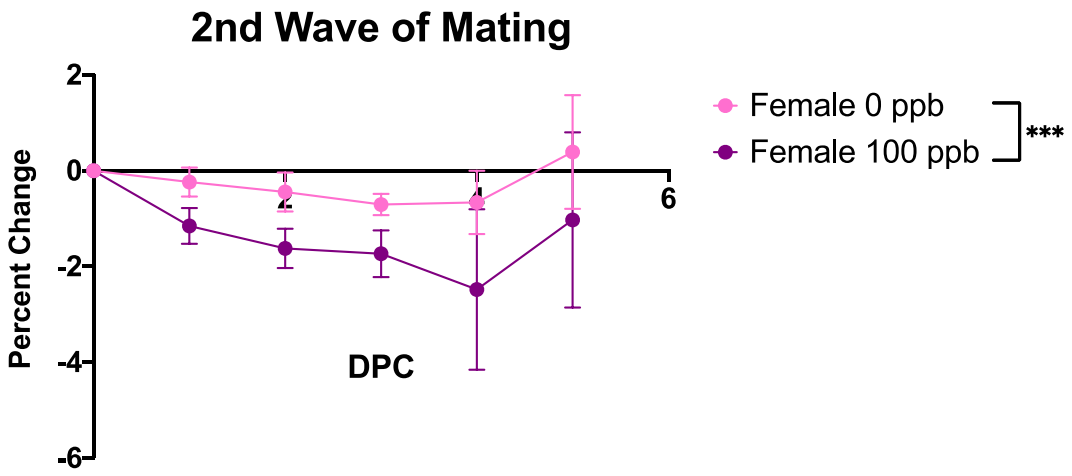

Figure 21: Percent temperature change days post challenge (DPC) for female offspring from the 2nd wave of mating. N=9-11 offspring from 4 to 5 litters; statistical significance indicated by ***$P < 0.001$.

4.4.3: Presentation of Clinical Signs

Following challenge with the drift variant ma2009 H1N1 IAV, morbidity was measured through the presence of clinical signs including piloerection, hunched posture, lack of an escape response, and dyspnea (Foltz and Ullman-Cullere 1999; Burkholder et al. 2012). Clinical signs were not observed for any of the vaccinated offspring from either the 1st or 2nd waves of mating.

PART 5: DISCUSSION

5.1: *In Utero* Arsenic Exposure and Birth Outcomes

In our experimental model, *in utero* exposure to arsenic did not result in significant changes to reproductive success, fetal resorptions, gestation length, or litter size compared to the control group (Table 6). These results coincide with a similar study that used C57BL/6 mice and found no difference in reproductive success, gestation length, or litter size following *in utero* exposure to arsenic at a concentration of 10 ppb (Kozul-Horvath et al. 2012). Our findings also match another study that acutely exposed LM/Bc/Fnn dams to high doses of arsenic (4.8-14.4 mg/kg) between gestation day 7 and 8, which resulted in no difference in both fetal resorptions and litter size (Hill, Wlodarczyk, and Finnell 2008).

Looking at pooled data from the 1st and 2nd waves of mating, *in utero* arsenic exposure resulted in a significant decrease in crown-to-rump length for male pups; weight of male pups, as well as weight and crown-to-rump length of female pups were also lower for the arsenic-exposed group but the difference was not statistically significant (Table 9). Our results concur with other *in vivo* studies looking at the effects of either chronic or acute *in utero* exposure to arsenic (Hood and Bishop 1972; Kozul-Horvath et al. 2012; Hill, Wlodarczyk, and Finnell 2008).

The results from our study, as well as similar *in vivo* studies looking at the effect of *in utero* arsenic exposure on birth outcomes does not correspond to findings from epidemiology studies looking at human populations exposed to arsenic. Studies conducted in Bangladesh have associated maternal and *in utero* arsenic exposure, at varying concentrations, with higher rates of spontaneous abortion and stillbirth, in addition to increased risk of preterm birth and low birth weight (Ahmad et al. 2001; Milton et al. 2005; Huyck et al. 2007). These same findings on the adverse effects of arsenic on birth outcomes and newborn health were also found in the United States, Taiwan, and Mongolia (Gilbert-Diamond et al. 2016; Yang et al. 2003; Myers et al. 2009).

In utero exposure to arsenic resulted in a significant decrease in fetal weight on gestation day 17, but no difference in placenta weight; this novel finding supports both *in vivo* and epidemiology studies that link arsenic to low birth weight (Table 10; Kozul-Harvath et al. 2012; Huyck et al. 2007). Although we did not find significant differences in heart or lung tissue weight at gestation day 17, other studies have linked *in utero* arsenic exposure to lower weight of male reproductive organs including the testes, seminal vesicle, and prostate (Table 10; Reddy et al. 2012).

The adverse reproductive effects of *in utero* arsenic exposure could be linked to the metalloid's ability to cause inflammation and oxidative stress. Results from a population-based study in Bangladesh indicated that *in utero*

arsenic exposure increased markers of oxidative damage in the placenta, as well as increased pro-inflammatory cytokines, including interleukin 1 beta and tumor necrosis factor alpha, in both placental tissue and cord blood (Ahmed et al. 2011). Supporting animal studies have investigated arsenic as a reproductive toxin and found that exposure to the metalloid during pregnancy increased fibrosis, hemorrhagic regions, and infarct lesions in the placenta. This same study observed a decrease in expression of glucose transporter type 4, which is an insulin-regulated transporter that plays an important role in maintaining the metabolic needs of the placenta during the early stages of pregnancy; this decrease in glucose transporter expression could be linked to fetal nutrition, and ultimately fetal weight and health (Gutierrez-Torres et al. 2015; Ericsson et al. 2005). Another animal study discovered that the metalloid increases maternal metallothionein levels in the liver, resulting in decreased zinc accumulation in the fetus; deficient levels of zinc during development is linked to embryotoxicity and can even lead to fetal death (Taubeneck et al. 1994; Golub, Macintosh, and Baumrind 1998).

Both animal and human studies have confirmed that arsenic readily crosses the placenta to reach the developing fetus. Concentrations of arsenic metabolites, including MMA and DMA, found in placental tissue significantly correlate with metabolites found in both cord blood and urine, as well as fetal organs such as the liver and brain (Hood et al. 1988; Jin et al. 2006; Concha

et al. 1998; Markowski et al. 2010). In addition, *in utero* arsenic exposure has been shown to alter lung size and mechanics, as well as up-regulate genes involved in mucus production and innate immunity (Ramsey et al. 2013c). Furthermore, *in utero* and postnatal exposure to arsenic has been linked to declines in infant health, measured through increased rates of bronchiectasis and respiratory tract infections (Smith et al. 2006; Farzan et al. 2013).

5.2: Effect of *In Utero* Arsenic on Influenza Vaccine Efficacy

Overall, results from our study indicate that *in utero* arsenic exposure does not adversely alter influenza vaccine efficacy in the offspring, evaluated through antibody titers, as well as body weight and temperature change following intranasal challenge. Looking at pre-challenge anti-ma2009 IAV IgG titers, arsenic-exposed offspring did not have lower titers compared to controls, demonstrating that arsenic, at a concentration of 100 ppb, does not change vaccine immunogenicity. Additionally, female offspring from both treatment groups appear to produce more anti-ma2009 IAV IgG compared to their male counterparts (Figures 4 and 5). There was also no difference in IgG1 to IgG2a/c titer ratios between the two treatment groups (Figures 6 and 7). Looking at neutralizing antibody titers from the 1st wave of mating, female offspring from the 100 ppb treatment group had significantly greater neutralizing capacity compared to female offspring from the 0 ppb treatment

group (Figures 8 and 9); the 1st wave of mating was immunized with 17.99 μg/dose for the first vaccination and 32.16 μg/dose for the second vaccination, while the 2nd wave of mating was immunized with 33.22 μg/dose for the first vaccination and 36.19 μg/dose for the second vaccination (Table 1).

By comparing pre-challenge immunogenicity endpoints including total anti-ma2009 IAV IgG titers, IgG1 to IgG2a/c titer ratios, and neutralizing antibody titers between the 1st and 2nd waves of mating, the results do not support the idea that vaccinating with a higher concentration of inactivated viral protein results in a greater antibody-mediated immune response. Generally, offspring from the 1st wave of mating initiated a more protective response following vaccination compared to offspring from the 2nd wave of mating, although not statistically significant.

Since arsenic is a known immunotoxin, it was hypothesized that *in utero* exposure to the metalloid would decrease influenza vaccine efficacy in the offspring (as reviewed by Dangleben, Skibola, and Smith 2013; World Health Organization 2018). In addition, because B cells of neonates and infant are undergoing development and have lower expression of T cell and complement receptors, as well as decreased somatic hypermutation when compared to adults, it results in a dampened humoral immune response and blunted affinity maturation of antibodies (as reviewed by Simon, Hollander, and McMichael 2015; Glaesener et al. 2018). In our study, we did not observe a diminished immune response following vaccination in both male and female

offspring exposed to arsenic *in utero*. This could possibly be due to the fact that the offspring were vaccinated during the juvenile life stage rather than during infancy, resulting in a more robust antibody-mediate immune response following vaccination. Although still undergoing development during childhood, the immune system shifts away from a strong Th2 response that is characteristic of development and infancy to favor a Th1 response upon antigen presentation (as reviewed by Simon, Hollander, and McMichael 2015; Hannet et al. 1992; Spellberg and Edwards 2001).

A previous epidemiology study has shown that exposure to arsenic does not adversely alter the immunogenicity of the inactivated cholera vaccine or the tetanus and diphtheria toxoid vaccines; this same study also found that arsenic did not effect the immunogenicity of the live-attenuated measles vaccine (Saha et al. 2013; Clem 2011; Centers for Disease Control and Prevention 2013). However, another epidemiology study has shown that early life exposure to arsenic can decrease the levels of mumps-specific IgG following immunization with the live-attenuated vaccine (Raqib et al. 2017). In our study, offspring exposed to arsenic *in utero* were vaccinated with an inactivated IAV vaccine. Pre-challenge titers showed no significant difference in immunogenicity between the arsenic-exposed offspring and the control offspring. Because of the results from Raqib et al., it suggests that the immunogenicity of live-attenuated vaccines may be more susceptible to the immunotoxic effects of arsenic. Using a live-attenuated IAV vaccine,

currently under development by Drs. Sabra Klein and Andrew Pekosz (Johns Hopkins Bloomberg School of Public Health), could yield different results that support arsenic's ability to adversely alter vaccine immunogenicity.

Overall, pre-challenge measurements of antibody quantity and functionality do not support the established concept that females mount a greater antibody-mediated immune response following vaccination. Both animal and human studies have demonstrated that biological sex plays a critical role in influenza vaccine efficacy. Although age and physiological factors including genetics and microbiome composition play a significant role, females tend to initiate higher antibody responses following immunization with the influenza vaccine (Klein and Pekosz 2014; Klein, Marriot, and Fish 2015). This is likely because of testosterone's immunosuppressive property, resulting in males having lower CD4 to CD8 ratios, producing decreased Th2 cytokine responses, and having lower levels of antigen-specific immunoglobulins following vaccination when compared to females (Furman et al. 2014; Klein, Marriot, and Fish 2015; Lorenzo et al. 2011). Our findings show that female offspring do not mount significantly greater antibody-mediated immune responses following vaccination when compared to male offspring. This is likely because the offspring were not sexually mature when they were vaccinated at 2 and 5 weeks of age, and therefore sex hormones like estrogen and testosterone did not play a key role in modulating the

immune response (Fink and Klein 2018; Jackson et al. 2017; The Jackson Laboratory 2016).

Because this was the first study to investigate how *in utero* arsenic exposure alters influenza vaccine efficacy in the offspring, one of our primary objectives was to test different vaccine concentrations to establish what dose is the most optimal, as well as to determine whether a higher concentration of vaccine elicited a greater antibody-mediated response. Typically, adults are vaccinated with 15 μg/dose of inactivated viral protein every influenza season, while naïve children between the age of 6 months to 8 years who have never received an influenza vaccine are immunized with 2 doses of vaccine at a concentration of 15 μg/dose (Centers for Disease Control and Prevention 2019b; Centers for Disease Control and Prevention 2017). In the adult ma2009 H1N1 IAV vaccine model established by Drs. Sabra Klein and Andrew Pekosz (Johns Hopkins Bloomberg School of Public Health), mice receive 2 doses of inactivated viral protein at a concentration of 20 μg/dose (Fink et al. 2018). Although the 2nd wave of mating was vaccinated with a higher concentration of inactivated viral protein, the offspring did not mount a greater response. This may possibly be due to a plateaued immune response following vaccination with a redundant amount of viral protein (Slifka and Amanna 2014).

Following challenge with the drift variant ma2009 H1N1 IAV, male and female offspring from both treatment groups did not experience a

significant drop in either weight or temperature, demonstrating the efficacy of the IAV vaccine (Figures 10, 13, 16, and 19). In addition, 0 ppb male offspring gained the greatest percentage of weight following challenge, followed by 100 ppb males, 0 ppb females, and 100 ppb females respectively (Figures 10 and 13). These findings correspond to various animal and human studies that have linked arsenic exposure with lower body weight compared to control groups (Kozul-Horvath et al. 2012; Hopenhayn et al. 2003; Nermell et al. 2008). Being underweight has been shown to be an important risk factor for both complications and hospitalizations associated with respiratory infections including influenza (Karki et al. 2018; Okubo et al. 2018; Moser et al. 2019).

Previous studies have established that arsenic is a developmental toxin, and that *in utero* exposure negatively affects lung development. *In utero* arsenic exposure not only causes a decrease in lung weight, but also reduces thoracic gas volume and increased lung tissue elastance (Ramsey et al. 2013b; Ramsey et al. 2013c; Petrick et al. 2009). Although we did observe signs of malaise in our study, altered lung structure and mechanics could lead to greater susceptibility to respiratory diseases, as well as higher morbidity and mortality (Ramsey et al. 2013c; Lantz et al. 2009).

PART 6: CONCLUSION

6.1: Key Findings

Arsenic is a common groundwater contaminant and millions of individuals consume drinking water with arsenic concentrations that far exceed the World Health Organization standard of 10 μg/L or ppb. Arsenic is a known carcinogen, immunotoxin, and endocrine disruptor, as well as a reproductive and developmental toxin (World Health Organization 2018).

Our findings show that *in utero* exposure to the metalloid, at a concentration of 100 ppb, has negative effects on both fetal and pup weight. Although we did not observe any differences in antibody-mediated immune responses following vaccination, nuanced differences may have been seen at lower vaccination concentrations that more accurately model the human vaccination schedule. Another important finding from the study is that *in utero* arsenic exposure results in lower body weight during adulthood, which could contribute to greater morbidity and mortality to respiratory infections like the influenza virus (Karki et al. 2018; Okubo et al. 2018; Moser et al. 2019).

6.2: Public Health Impact

This study was the first to look at the effects of *in utero* arsenic exposure on juvenile IAV vaccine immunogenicity and subsequent infection

susceptibility during adulthood. The results from this study indicate that exposure to the metalloid throughout pregnancy does not negatively alter the efficacy of the inactivated influenza vaccine in the offspring. These findings are promising for public health since they suggest that individuals exposed to arsenic *in utero* can be well-protected from influenza-related morbidity and mortality by vaccination with an inactivated IAV vaccine.

6.3: Future Directions

Preliminary data collected from the gestation day 17 euthanasia show a trend, albeit not significant, between *in utero* arsenic exposure and decreased fetal weight and crown-to-rump length, as well as a reduction in weight of fetal organs including the lungs and heart. Future efforts should be directed towards replicating this experimental design to strengthen this association. In addition, arsenic speciation should be compared between maternal tissue and fetal tissue to quantify how much arsenic and what metabolites are transferred from mother to fetus. Focusing on the placenta, gene and protein expression should also be investigated to look at changes in activity of key nutrient transporters including the sodium-coupled neutral amino acid transporter and the fatty acid transport protein which provide channels for the movement of nutrients from mother to fetus (Brett et al. 2014; Desforges et al. 2010).

Another future direction of this project is to alter the vaccine concentrations used to immunize the offspring. The results showing immunogenicity of the vaccine demonstrate a plateau effect for the 2nd wave of mating since the offspring overall produced a lower antibody-mediated response compared to the 1st wave of mating. By using a lower vaccine concentration, subtle differences in anti-ma2009 IAV IgG titers or neutralizing antibody titers, modulated by *in utero* arsenic exposure, may become more evident. In addition, instead of using inactivated viral protein for intraperitoneal immunization, the offspring could be intransally vaccinated with a live-attenuated vaccine that is currently under development. Live-attenuated vaccines elicit a different immune response compared to inactivated vaccines; live-attenuated vaccines are characterized by high IgA and IgM levels in the mucosal tissues of the respiratory tract following immunization whereas inactivated vaccines are primarily correlated with high levels of serum IgG (Cox, Brokstad, and Ogra 2004; Chung et al. 2019). Because of the difference in immune response, the live-attenuated vaccine may be more sensitive to the immunotoxic effects associated with arsenic exposure.

Previous work looking at the effects of chronic arsenic exposure and influenza vaccine efficacy conducted by Sarah Attreed, a current doctoral student, has shown that the metalloid significantly increases pro-inflammatory cytokines and chemokines including interleukin 9, interleukin

18, and monocyte chemoattractant protein 1 following both vaccination and intranasal challenge. Pro-inflammatory cytokines increase disease severity, and play an important role in response to infection and tissue damage (Van Reeth 2000; Sladkova and Kostolansky 2006; Betakova et al. 2017; Dinarello 2000). Because of these previous findings in an adult mouse model, future work should be directed towards looking at pro-inflammatory cytokines and chemokines in lung homogenates of vaccinated offspring exposed to arsenic *in utero*.

In addition to looking at pro-inflammatory cytokines, lung homogenates can also be used to look at viral titers. Previous work by Sarah Attreed, as well as work by Kozul et al. and Ramsey et al. has shown increased viral load for unvaccinated adult mice chronically exposed to arsenic; higher viral titers indicate greater viral replication and are correlated with more severe symptoms (Kozul et al. 2009; Ramsey et al. 2013a; Koopman et al. 2016; Chen et al. 2012;). Although phenotypic measurements including body weight and temperature did not indicate significant morbidity following intranasal challenge, it would be beneficial to measure viral titers in lung homogenates to look for differences in viral replication and clearance between the experimental and control offspring (Lee et al. 2009; Baccam et al. 2006).

Following challenge with the drift variant ma2009 H1N1 IAV, tissues including lungs, spleen, thymus, and mediastinal lymph nodes were collected

and cryopreserved for future work. Another important direction is to look at these tissue samples using flow cytometry to see if there are differences in immune cell populations between exposed and unexposed offspring; important cell populations to quantify include antigen-specific T cells, as well as memory B cells (Henry et al. 2019; Powell et al. 2013; Schulze-Horsel, Genzel, and Reichl 2008). In addition, important markers of influenza infection including viral nucleoprotein and matrix protein 1 could be investigated using gene and protein expression (Schulze-Horsel, Genzel, and Reichl 2008; Biswas, Boutz, and Nayak 1998).

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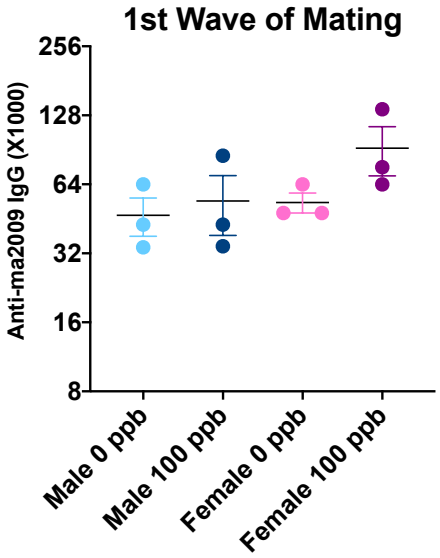
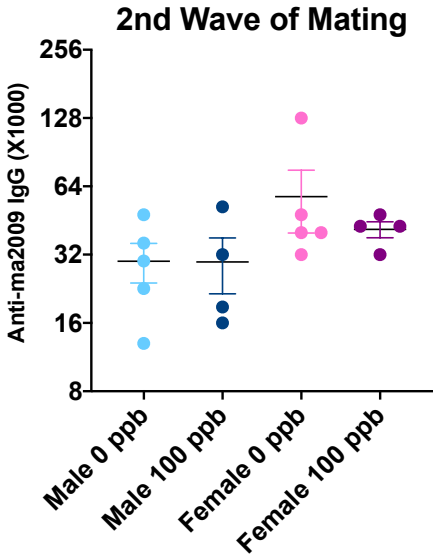
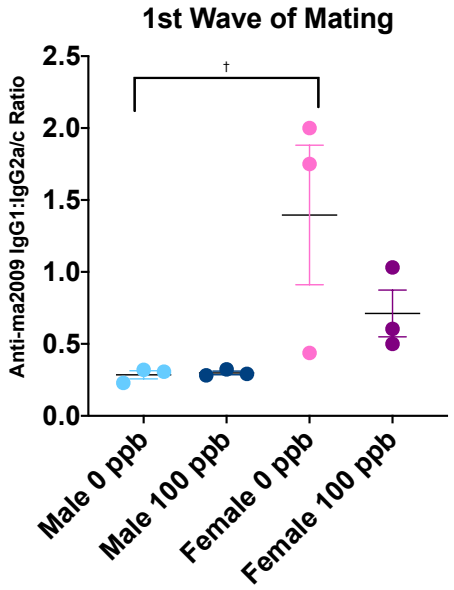
Supplemental Information



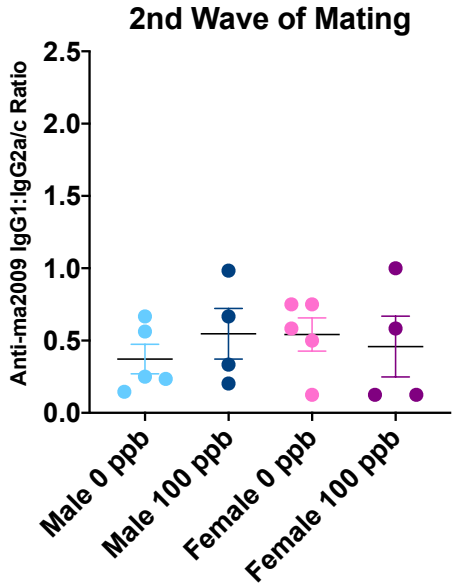
Supplemental Figure 1: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG titers for the 1st wave of mating, graphed by litter average. Male and female offspring were immunized with 17.99 µg/dose for vaccination 1 at 2 weeks of age, and 33.22 µg/dose for vaccination 2 at 5 weeks of age. N=3 litters, with the number of pups per data point ranging from 1 to 8; no significance.



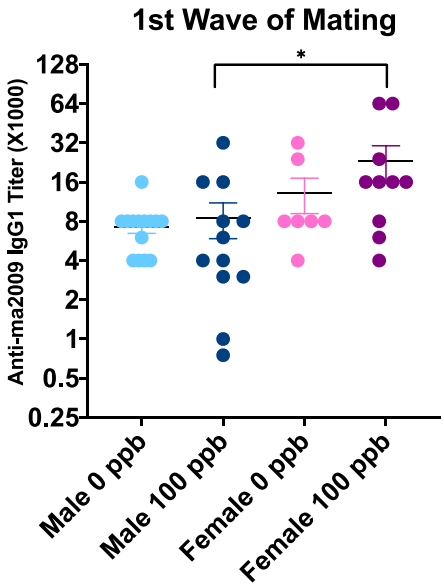
Supplemental Figure 2: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG titers for the 2nd wave of mating, graphed by litter average. Male and female offspring were immunized with 32.16 µg/dose for vaccination 1 at 2 weeks of age, and 36.19 µg/dose for vaccination 2 at 5 weeks of age. N=4-5 litters with the number of pups per data point ranging from 1 to 6; no significance.



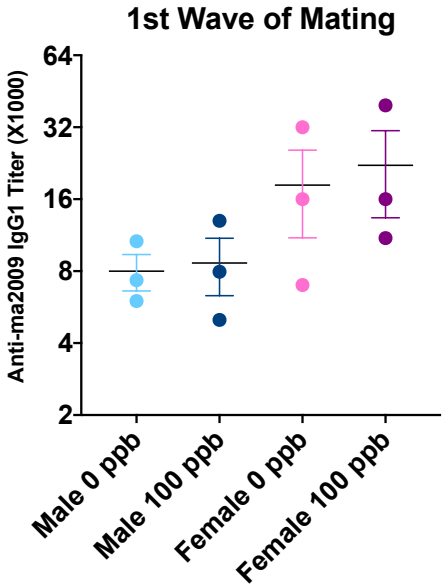
Supplemental Figure 3: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG1:IgG2a/c titer ratios for the 1st wave of mating, graphed by litter average. Male and female offspring were immunized with 17.99 µg/dose for vaccination 1 at 2 weeks of age, and 33.22 µg/dose for vaccination 2 at 5 weeks of age. N=3 litters, with the number of pups per data point ranging from 1 to 8; $P < 0.10$ indicated by †.



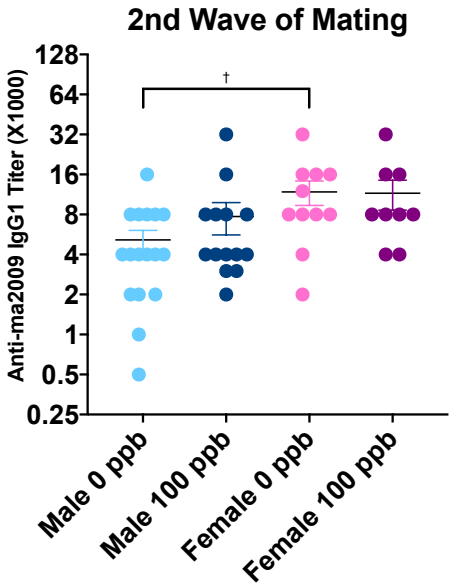
Supplemental Figure 4: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG1:IgG2a/c titer ratios for the 2nd wave of mating, graphed by litter average. Male and female offspring were immunized with 32.16 µg/dose for vaccination 1 at 2 weeks of age, and 36.19 µg/dose for vaccination 2 at 5 weeks of age. N=4-5 litters with the number of pups per data point ranging from 1 to 6; no significance.



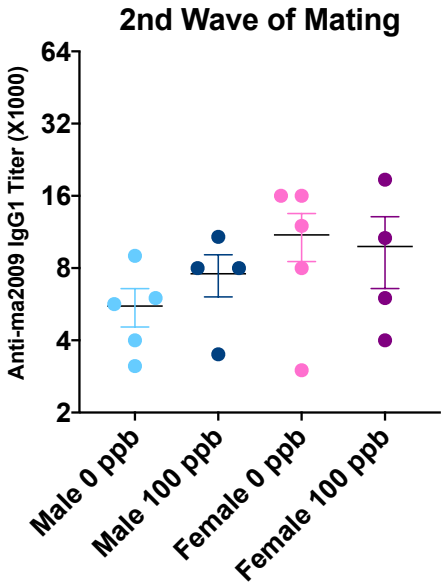
Supplemental Figure 5: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG1 titers for the 1st wave of mating. Male and female offspring were immunized with 17.99 µg/dose for vaccination 1 at 2 weeks of age, and 33.22 µg/dose for vaccination 2 at 5 weeks of age. N=7-14 offspring from 3 litters; statistical significance indicated by $*P < 0.05$.



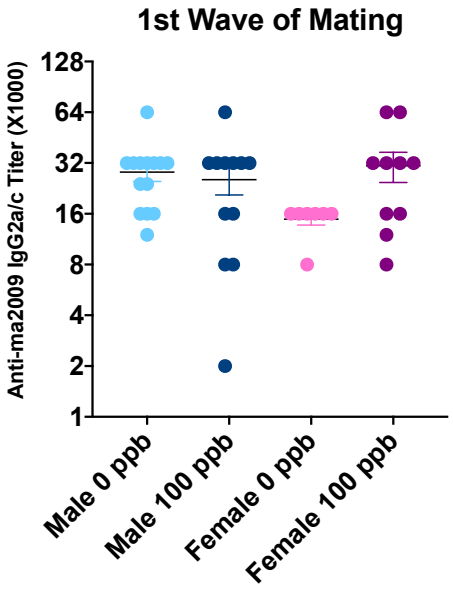
Supplemental Figure 6: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG1 titers for the 1st wave of mating, graphed by litter average. Male and female offspring were immunized with 17.99 µg/dose for vaccination 1 at 2 weeks of age, and 33.22 µg/dose for vaccination 2 at 5 weeks of age. N=3 litters, with the number of pups per data point ranging from 1 to 8; no significance.



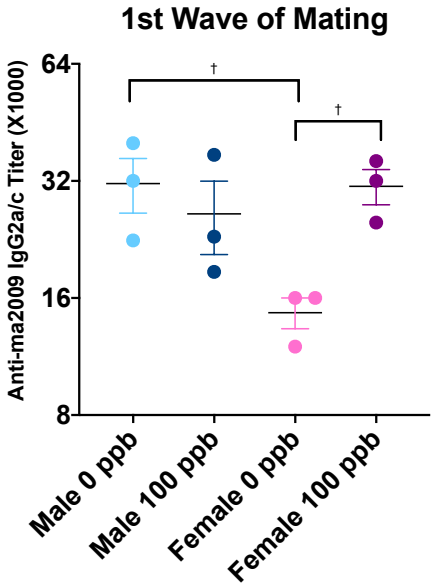
Supplemental Figure 7: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG1 titers for the 2nd wave of mating. Male and female offspring were immunized with 32.16 µg/dose for vaccination 1 at 2 weeks of age, and 36.19 µg/dose for vaccination 2 at 5 weeks of age. N=9-17 offspring from 4 to 5 litters; $P < 0.10$ indicated by †.



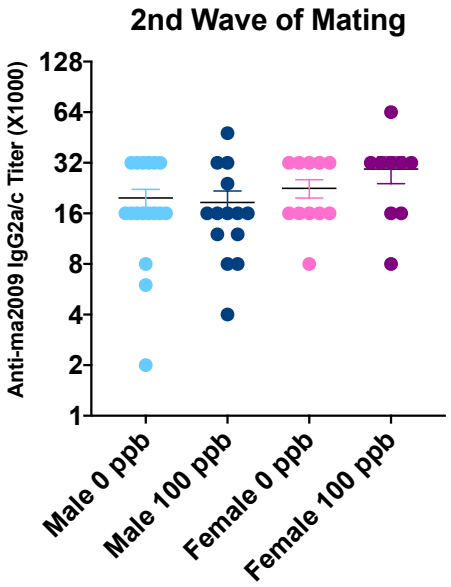
Supplemental Figure 8: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG1 titers for the 2nd wave of mating, graphed by litter average. Male and female offspring were immunized with 32.16 µg/dose for vaccination 1 at 2 weeks of age, and 36.19 µg/dose for vaccination 2 at 5 weeks of age. N=4-5 litters with the number of pups per data point ranging from 1 to 6; no significance.



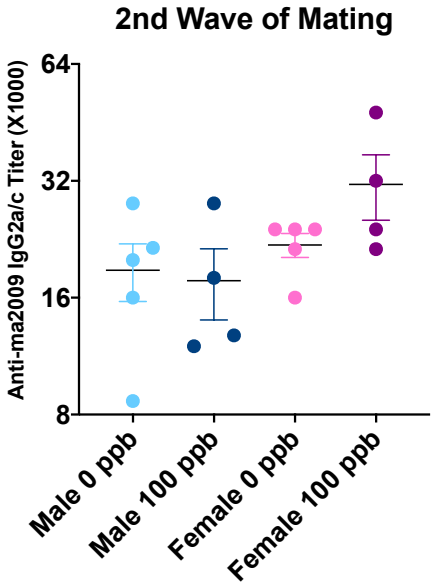
Supplemental Figure 9: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG2a/c titers for the 1st wave of mating. Male and female offspring were immunized with 17.99 µg/dose for vaccination 1 at 2 weeks of age, and 33.22 µg/dose for vaccination 2 at 5 weeks of age. N=7-14 offspring from 3 litters; no significance.



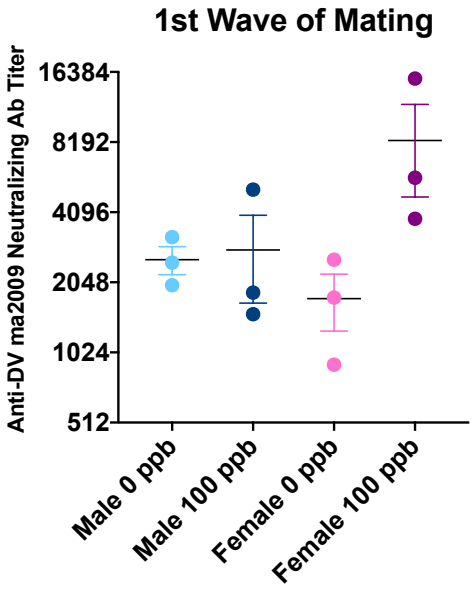
Supplemental Figure 10: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG2a/c titers for the 1st wave of mating, graphed by litter average. Male and female offspring were immunized with 17.99 µg/dose for vaccination 1 at 2 weeks of age, and 33.22 µg/dose for vaccination 2 at 5 weeks of age. N=3 litters, with the number of pups per data point ranging from 1 to 8; $P < 0.10$ indicated by †.



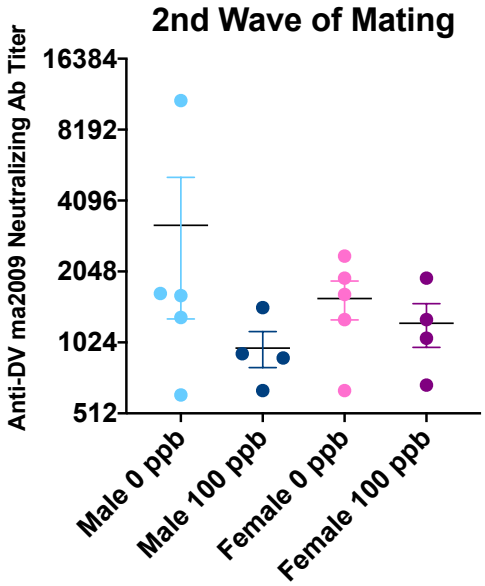
Supplemental Figure 11: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG2a/c titers for the 2nd wave of mating. Male and female offspring were immunized with 32.16 µg/dose for vaccination 1 at 2 weeks of age, and 36.19 µg/dose for vaccination 2 at 5 weeks of age. N=9-17 offspring from 4 to 5 litters; no significance.



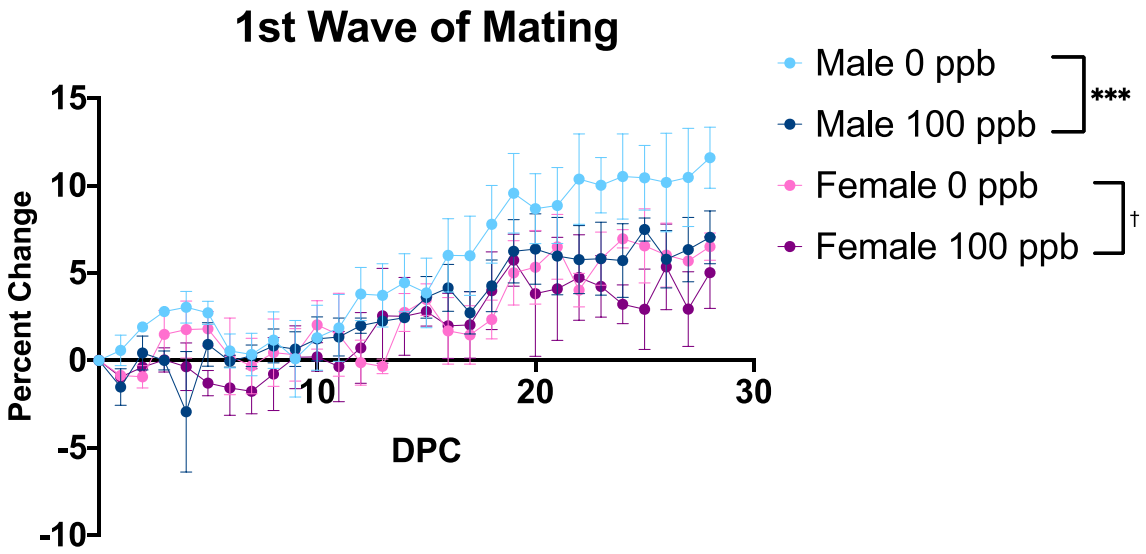
Supplemental Figure 12: Pre-challenge (21 days post vaccination 2) anti-ma2009 IAV IgG2a/c titers for the 2nd wave of mating, graphed by litter average. Male and female offspring were immunized with 32.16 µg/dose for vaccination 1 at 2 weeks of age, and 36.19 µg/dose for vaccination 2 at 5 weeks of age. N=4-5 litters with the number of pups per data point ranging from 1 to 6; no significance.



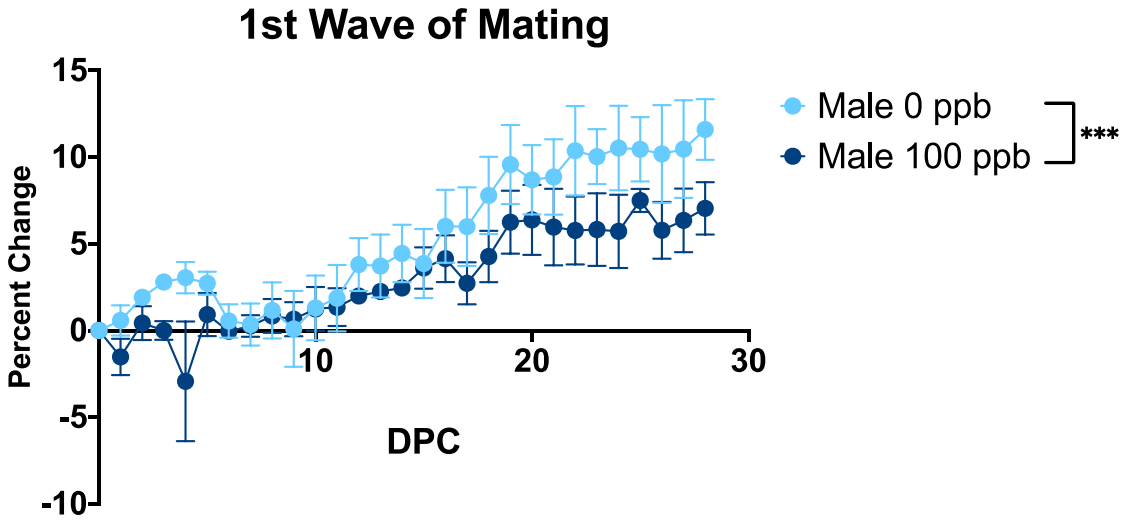
Supplemental Figure 13: Pre-challenge (21 days post vaccination 2) anti-DV ma2009 IAV neutralizing antibody titers for the 1st wave of mating, graphed by litter average. Male and female offspring were immunized with 17.99 µg/dose for vaccination 1 at 2 weeks of age, and 33.22 µg/dose for vaccination 2 at 5 weeks of age. N=3 litters, with the number of pups per data point ranging from 1 to 8; no significance.



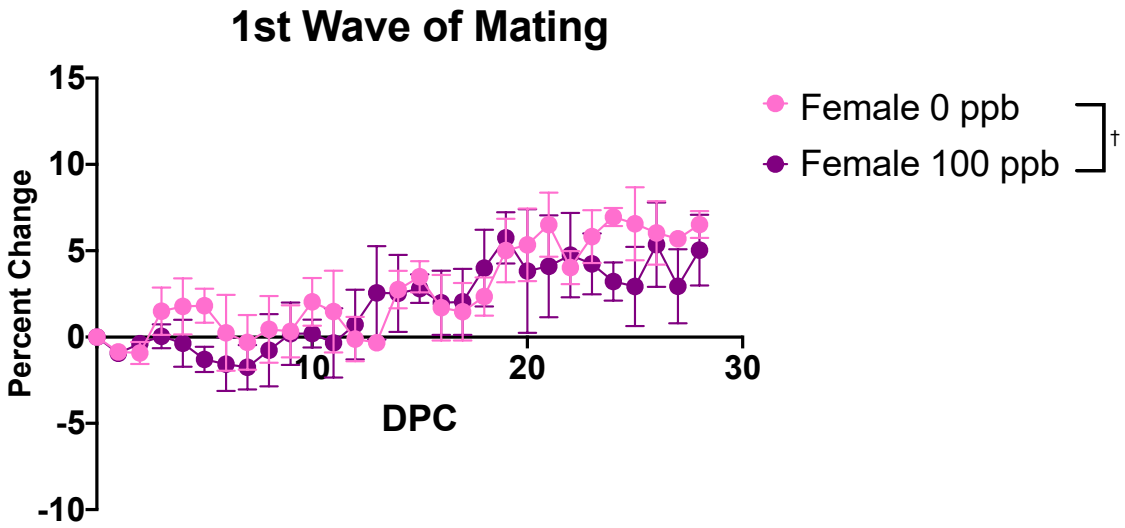
Supplemental Figure 14: Pre-challenge (21 days post vaccination 2) anti-DV ma2009 IAV neutralizing antibody titers for the 2nd wave of mating, graphed by litter average. Male and female offspring were immunized with 32.16 µg/dose for vaccination 1 at 2 weeks of age, and 36.19 µg/dose for vaccination 2 at 5 weeks of age. N=4-5 litters with the number of pups per data point ranging from 1 to 6; no significance.



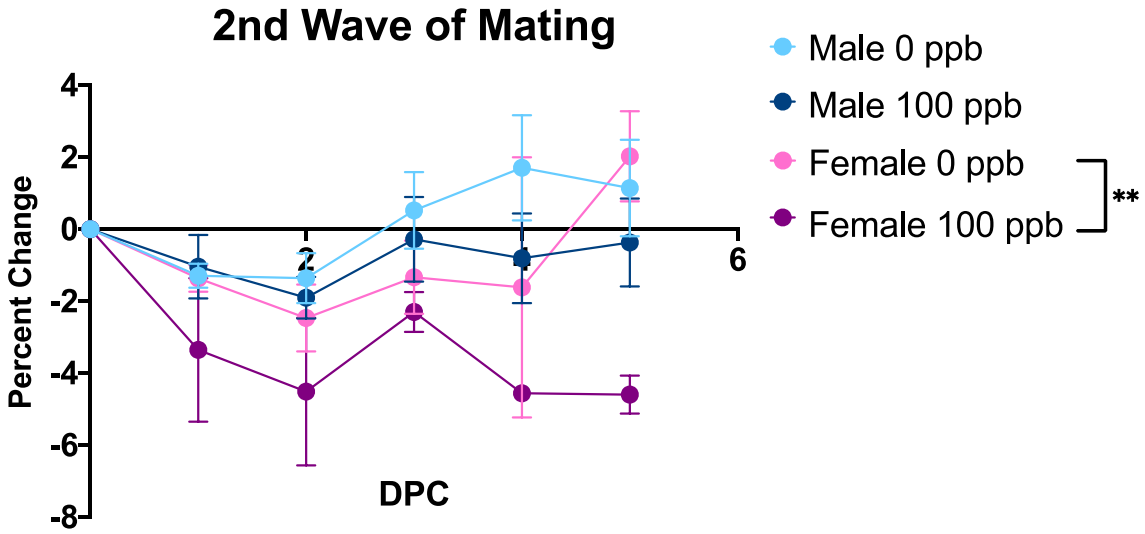
Supplemental Figure 15: Percent body weight change days post challenge (DPC) for offspring from the 1st wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=3 litters, with the number of pups per data point ranging from 1 to 5; $P < 0.10$ indicated by † and statistical significance indicated by ***$P < 0.001$.



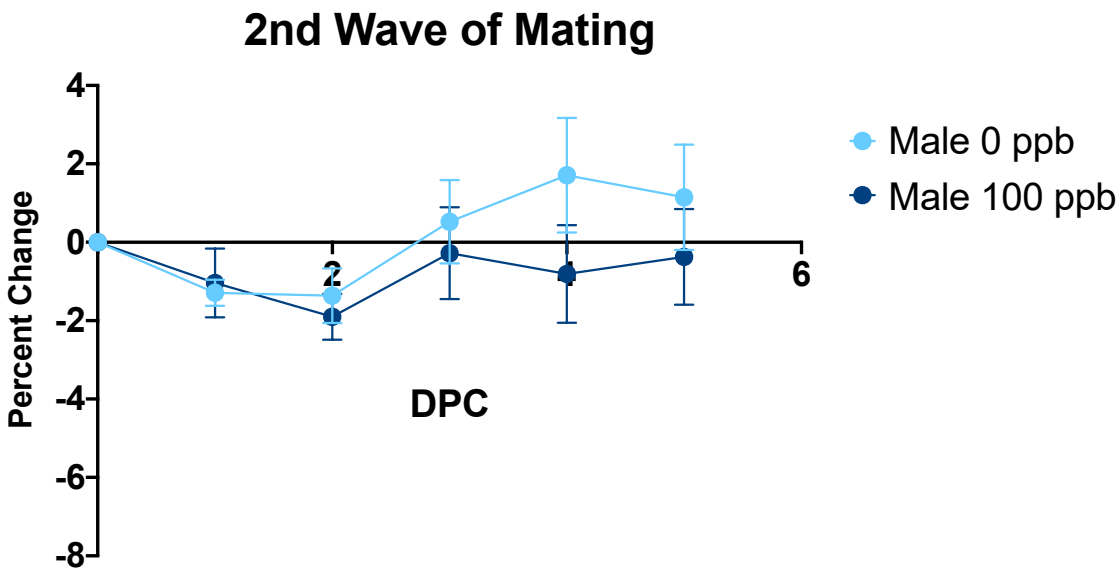
Supplemental Figure 16: Percent body weight change days post challenge (DPC) for male offspring from the 1st wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=3 litters, with the number of pups per data point ranging from 2 to 5; statistical significance indicated by ***$P < 0.001$.



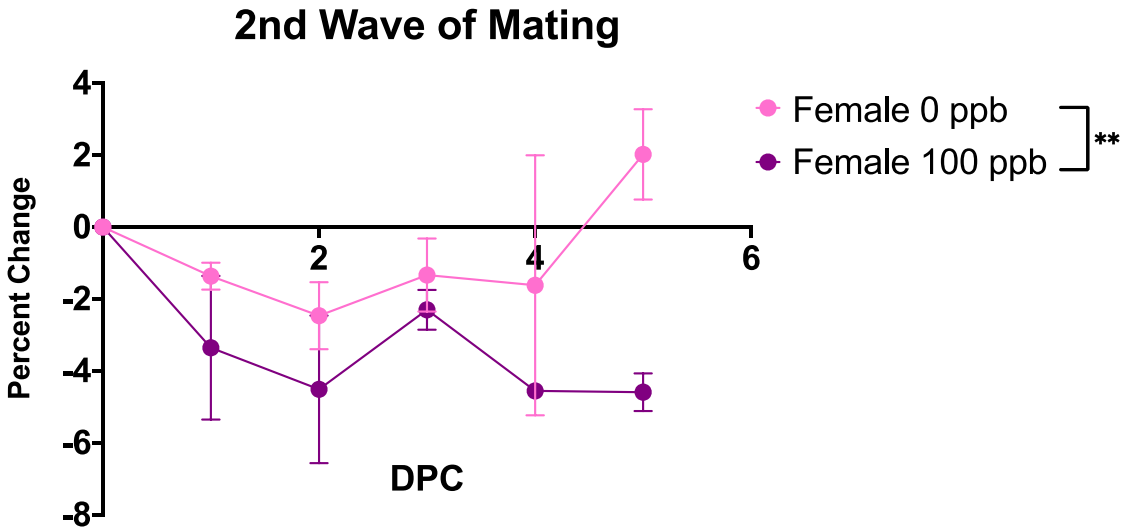
Supplemental Figure 17: Percent body weight change days post challenge (DPC) for female offspring from the 1st wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=3 litters, with the number of pups per data point ranging from 1 to 3; $P < 0.10$ indicated by †.



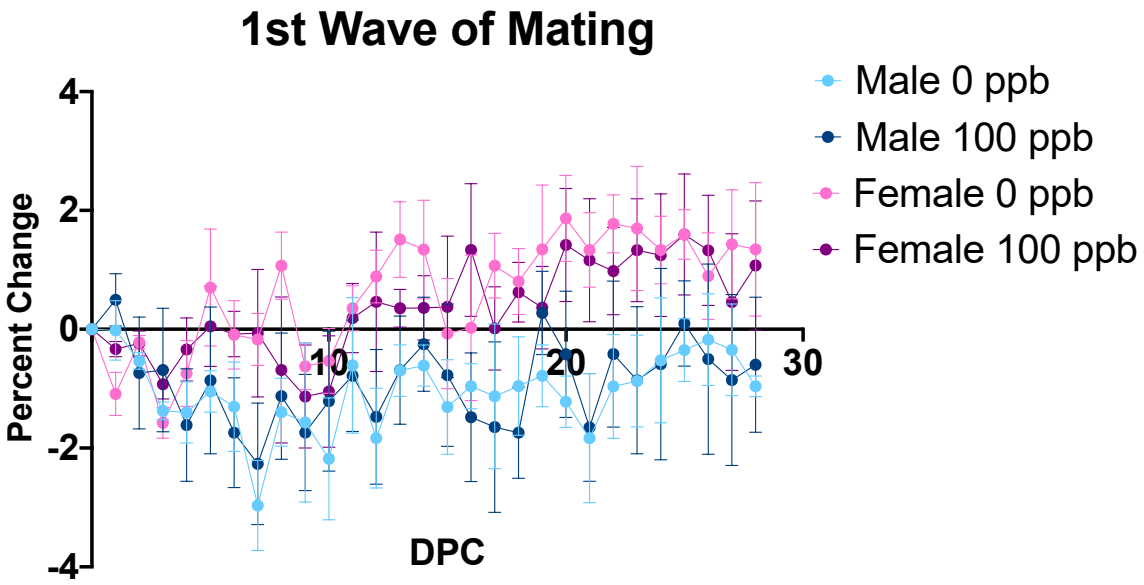
Supplemental Figure 18: Percent body weight change days post challenge (DPC) for offspring from the 2nd wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=4-5 litters with the number of pups per data point ranging from 1 to 5; statistical significance indicated by $^{**}P < 0.01$.



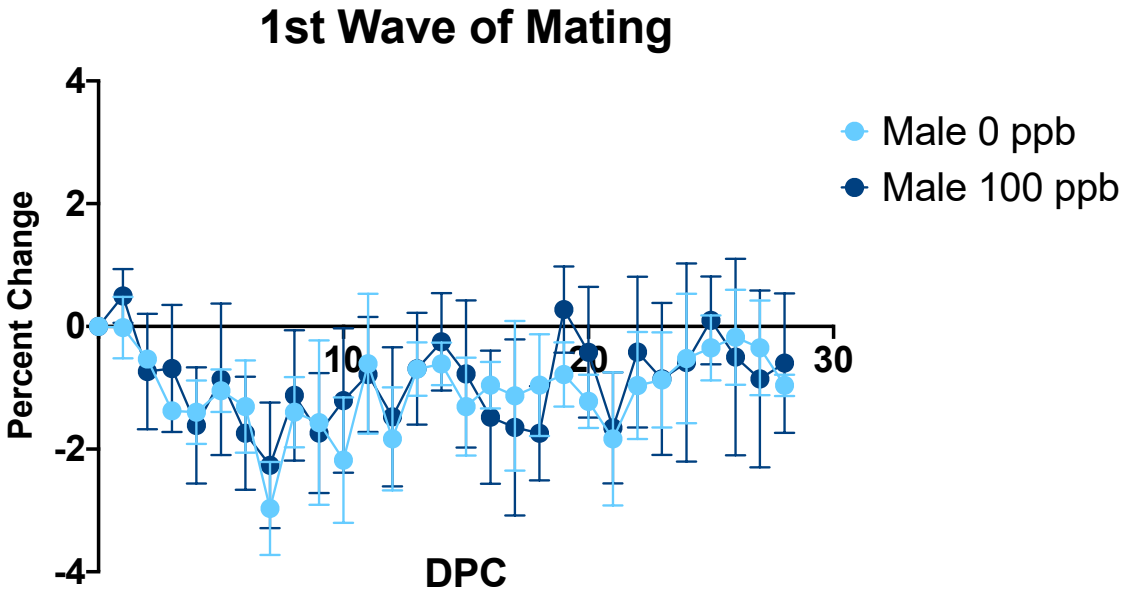
Supplemental Figure 19: Percent body weight change days post challenge (DPC) for male offspring from the 2nd wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=4-5 litters with the number of pups per data point ranging from 1 to 5; no significance.



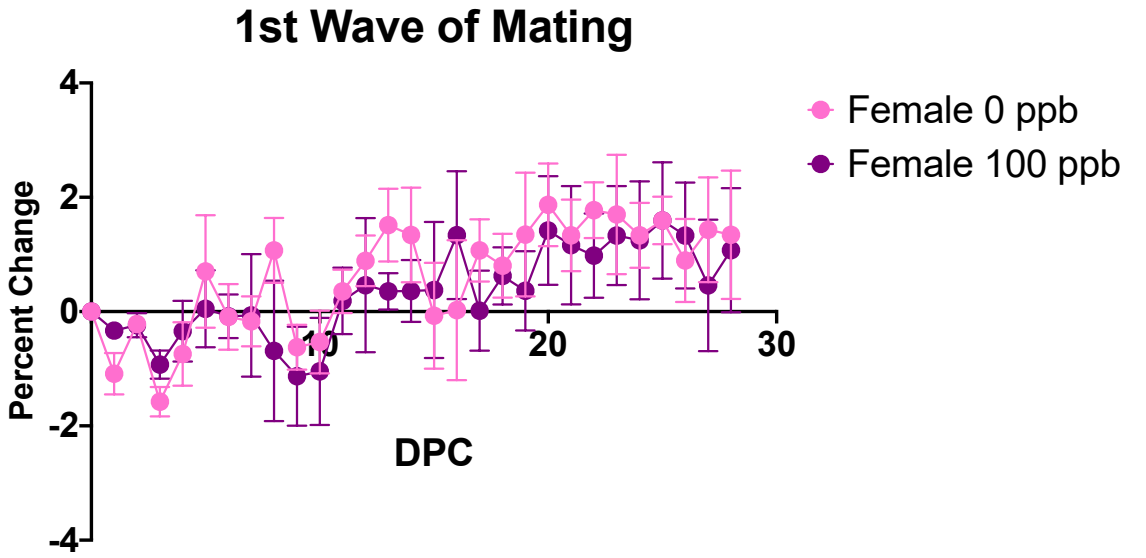
Supplemental Figure 20: Percent body weight change days post challenge (DPC) for female offspring from the 2nd wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=4-5 litters with the number of pups per data point ranging from 1 to 3; statistical significance indicated by $^{**}P < 0.01$.



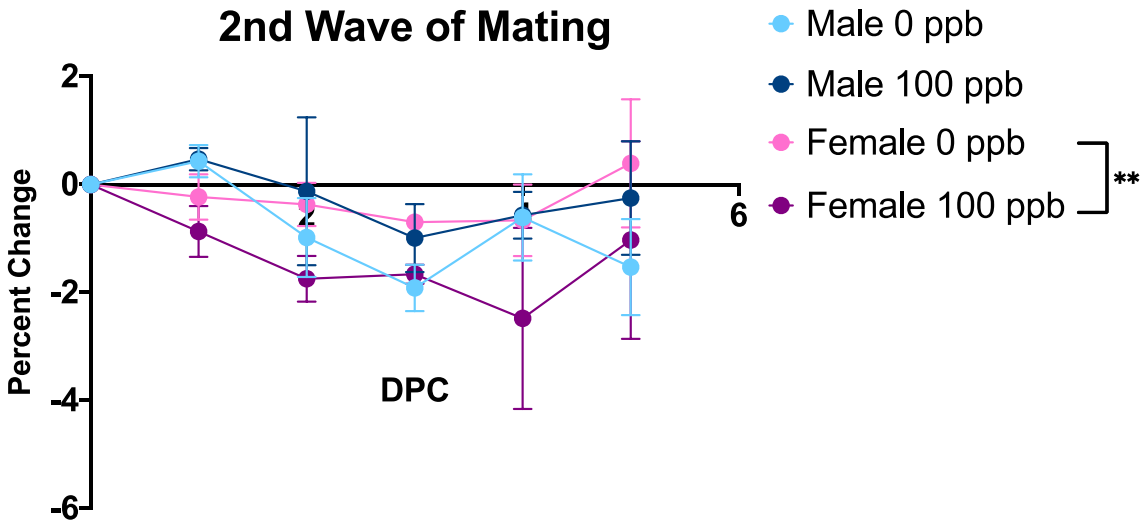
Supplemental Figure 21: Percent temperature change days post challenge (DPC) for offspring from the 1st wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=3 litters, with the number of pups per data point ranging from 1 to 5; no significance.



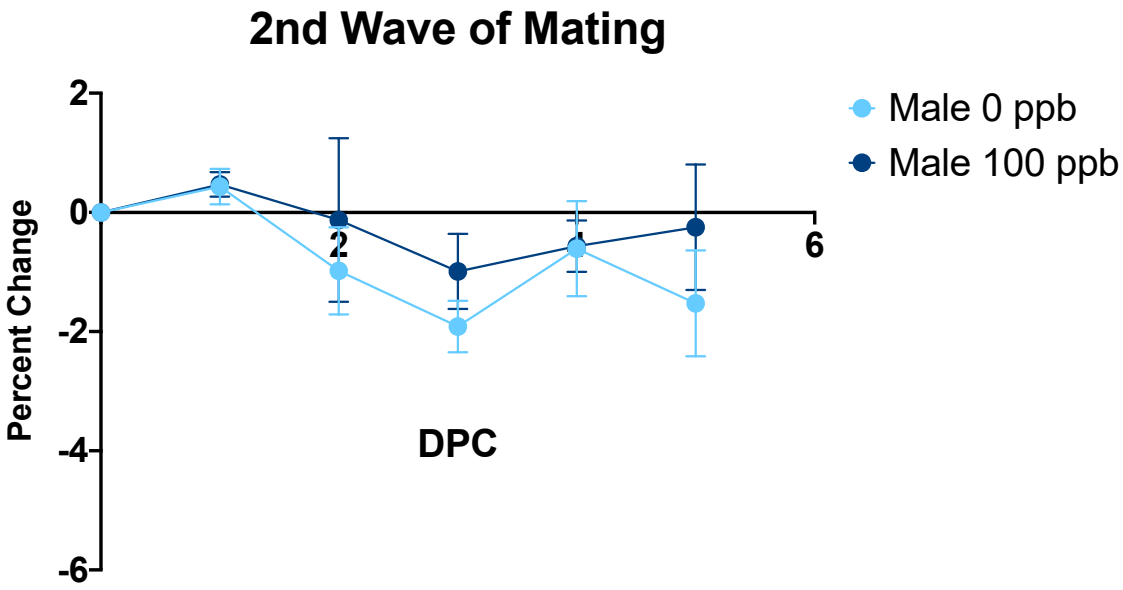
Supplemental Figure 22: Percent temperature change days post challenge (DPC) for male offspring from the 1st wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=3 litters, with the number of pups per data point ranging from 2 to 5; no significance.



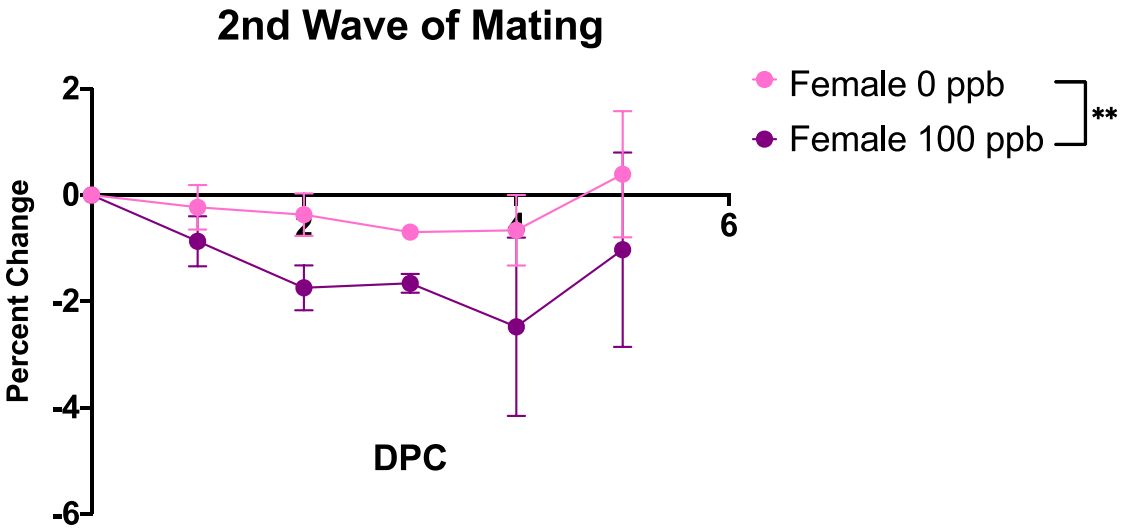
Supplemental Figure 23: Percent temperature change days post challenge (DPC) for female offspring from the 1st wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=3 litters, with the number of pups per data point ranging from 1 to 3; no significance.



Supplemental Figure 24: Percent temperature change days post challenge (DPC) for offspring from the 2nd wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=4-5 litters with the number of pups per data point ranging from 1 to 5; statistical significance indicated by $^{**}P < 0.01$.



Supplemental Figure 25: Percent temperature change days post challenge (DPC) for male offspring from the 2nd wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=4-5 litters with the number of pups per data point ranging from 1 to 5; no significance.



Supplemental Figure 26: Percent temperature change days post challenge (DPC) for female offspring from the 2nd wave of mating, graphed by litter average. At 8 weeks of age, offspring were intranasally challenged with a lethal dose of 10^5 tissue culture infectious dose of the drift variant ma2009 H1N1 IAV. N=4-5 litters with the number of pups per data point ranging from 1 to 3; statistical significance indicated by $^{**}P < 0.01$.

Curriculum Vitae

Chloe M. Kashiwagi

Sacramento, CA **I** 916-208-7080 **I** c.kashiwagi95@gmail.com

Professional Interests

As a graduate student with a strong interest in toxicology, I aspire to apply my academic training and laboratory experience to advance scientific research related to the impacts of the environment on human health. The rates of many diseases are on the rise, and I am passionate to investigate the association between exposure and outcome, as well as develop therapies for remediation.

Education

Johns Hopkins Bloomberg School of Public Health August 2017-May 2019
Master of Science (ScM) in Environmental Health and Engineering GPA: 3.95

- Focus in Toxicology, Pathophysiology, and Molecular Mechanisms
- Certificate in Risk Sciences and Public Policy

University of California, Berkeley August 2013-May 2017
Bachelor of Science in Molecular Environmental Biology GPA: 3.46

- Minor in Molecular Toxicology

Research Experience

Sillé Lab - Johns Hopkins School of Public Health January 2018-Present
Graduate Student Researcher

- Formulated and executed a project to study the immunotoxicological effects of arsenic exposure on influenza vaccine efficacy
- Extensive experience with experimental design, data collection, and statistical analysis

Almeida Lab - University of California, Berkeley August 2015-May 2017
Undergraduate Researcher

- Successfully completed a thesis project to study the transmission of Grapevine leafroll-associated virus 3, an agriculturally-significant grape pathogen
- Chosen to mentor and provide academic guidance for research projects of summer foreign exchange students

COSMOS School for Mathematics and Science - University of California, San Diego July 2012
Student Researcher

- Through a competitive application process, selected to participate in a summer research program sponsored by the Jacobs School of Engineering
- Completed a bioengineering-based project to understand how the mechanical structure of the red blood cell influences the development of sickle cell anemia

Laboratory Skills

- Animal Handling
- Dissection Techniques
- Immunoassays
- Mammalian Cell Culture
- DNA and RNA Extractions
- PCR and Gel Electrophoresis
- SDS-PAGE and Western Blot
- Next-Generation Sequencing

Publication

Prator, C.A., **Kashiwagi, C.M.**, Voncina, D., Almeida, R.P.P. 2017. Infection and Colonization of *Nicotiana benthamiana* by *Grapevine leafroll-associated virus 3. Virology* 510: 60-66.

Presentation

Investigating the Effects of In Utero Arsenic Exposure on Birth Outcomes and Influenza Vaccine Efficacy in the Offspring. Society of Toxicology: Immunotoxicology Poster Session. Baltimore, MD. March 2019.

Using a Mouse Model to Understand How In Utero Arsenic Exposure Affects Influenza A Virus Vaccine Efficacy. Johns Hopkins Bloomberg School of Public Health: Vaccine Day. Baltimore, MD. April 2018.

Teaching and Leadership

Graduate Student Teaching Assistant August-December 2018

- Selected as a teaching assistant for Public Health Toxicology, a graduate level course
- Facilitated discussions between faculty and students about toxicology concepts
- Actively involved in one-on-one tutoring with students, and grading assignments and exams

Johns Hopkins School of Public Health Student Ambassador June 2018-Present

- Acting as a student representative of Johns Hopkins Bloomberg School of Public Health
- Mentoring prospective students interesting in applying to the school
- Engaging in compelling conversations about the importance of public health

Environmental Health & Engineering Student Organization June 2018-Present

- Elected by faculty and students as Master program representative
- Responsible for the planning and execution of community service events and professional development workshops

Cal Alumni Student Association August 2013-May 2017

- Active participant in club activities including professional workshops, a student mentoring program, and Overnight Stay Program for incoming freshman

Awards

Centennial Scholars Fund Award January 2018

- Chosen to receive a merit-based scholarship from Johns Hopkins Centennial Scholars Program for promising and dedicated students in the field of public health

Cal Alumni Association Leadership Award Recipient August 2013

- Selected as top six percent of applicants to receive an academic scholarship from the Cal Alumni Association for outstanding leadership and dedication to the community

Girl Scout Gold Award June-August 2012

- Completed over 85 hours of volunteer work for My Sister's House, a non-profit organization that aids Asian and Pacific Islander women who are victims of domestic violence